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The Toxicology of Nitrates and Nitrites with Particular Reference to the Potability of Water Supplies

A Review*

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INTRODUCTION

THERE have been many reports in recent years that high concentrations of nitrates in drinking supplies have caused poisoning and death among infants and also among animals. A review has been made of the evidence available in order to suggest suitable limits for this constituent in drinking water supplies.

THE TOXICOLOGY OF NITRATES AND NITRITES

MODE OF ACTION—

After ingestion, nitrates are converted to nitrites by bacterial reduction. According to Cornblath and Hartmann,[1] this reduction occurs in the lower intestine of the adult. However, in very young babies, which have a physiological gastric achlorhydria, reduction occurs in the stomach and duodenum from which the nitrites are more readily absorbed into the blood stream. After absorption, nitrites convert oxyhaemoglobin into methaemoglobin and thus interfere with the oxygen transport in the blood. This effect may be cumulative, since the methaemoglobin is reduced only at a slow rate.

* Reprints of this paper will be available shortly. For details, please see p. 492.

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SYMPTOMS OF POISONING—

Luff[2] describes the symptoms of acute potassium nitrate poisoning. They occur in the order: (*a*) pain in the epigastrium; (*b*) purging, often with blood; (*c*) convulsions of the face muscles; (*d*) weak irregular pulse; (*e*) difficult respiration; (*f*) cold extremities followed by collapse. Cyanosis is usually observed and the blood becomes chocolate coloured.

ANTIDOTE—

Intravenous injection of methylene blue results in a rapid reduction of methaemoglobin to haemoglobin, and the patient makes a dramatic recovery.[3]

LETHAL DOSE: HUMAN—

Nitrates—Smith and Simpson[4] consider the normal fatal dose of potassium nitrate to be 15 to 30 g for an adult, although they quote a report by Peterson, Haines and Webster[5] of the death of an adult after taking 8 g. Luff[2] records the death of an adult after taking 3·5 g of potassium nitrate, although Witthaus[6] reports a recovery after taking 125 g. Comly[7] quotes the observation of Eusterman and Keith[8] that certain adults are extremely allergic to nitrates. These authors record that one adult suffered from extreme cyanosis after taking 7 g of ammonium nitrate daily for 8 successive days. Wood[9] also states that nitrates are a cumulative poison. On the other hand, Amberg[9] reports that a 13-year-old boy (110 lb) with glomerulonephritis took 6 g of potassium nitrate daily for a year without injury. Windmueller[10] states that an adult who took 4 teaspoonsful daily of an equal mixture of sulphur and potassium nitrate for 26 days died 17 days later.

Nitrites—Smith and Simpson[4] consider that the normal fatal dose of sodium nitrite is about 10 g. Manicatide[11] records the death of a 3-month-old child after taking 0·35 g of sodium nitrite, but the recovery of a 2-month-old child after taking 0·4 g. Tankard[12] reports the death of an adult who probably took about 7 g of sodium nitrite. Scholes[13] records the accidental death of three adults, two of them within an hour, after eating a meal seasoned with sodium nitrite instead of sodium chloride. Several other deaths from nitrite poisoning have been recorded.[14,15,16,17,18] However, Kobert, quoted by Autenrieth,[19] mentions the case of a patient who took "several doses" of 0·5 g of sodium nitrite at hourly intervals "without dangerous symptoms," although the symptoms included nausea, diarrhoea, cyanosis and increased diuresis.

Naidu and Venkatrao[20] consider 2·0 g of sodium nitrite to be a fatal dose for a 10-stone man after examining results obtained on dogs. This is equivalent to 32 mg per kg.

FATAL DOSE: ANIMALS—

Crawford[21] reports a case of sodium nitrate poisoning in cattle; he considers that ½ lb of sodium nitrate is a fatal dose. For an animal weighing 500 kg this is equivalent to 450 mg per kg. Bradley, Eppson and Beath[22,23] have shown that "oat hay poisoning" in cattle is caused by abnormally high concentrations of nitrate in the affected hay. They have also found that 1 g of potassium nitrate per kg is a lethal dose for cattle. Death is caused by methaemoglobinaemia, 70 to 80 per cent. of the haemoglobin being converted to methaemoglobin in a few hours. Sheep are similarly affected. Sapiro, Hoflund, Clark and Quin[24] have demonstrated that nitrate is reduced to nitrite by the rumen contents of cattle.

Lewis[25] has shown that, in the rumen of sheep, nitrate is reduced successively to nitrite and ammonia. With large doses of nitrate, the conversion of nitrite to ammonia is limited and the nitrite concentration in the rumen increases rapidly. After giving large doses of nitrate, the methaemoglobin in the blood is found to reach a maximum about 7 hours later. Lewis finds that the doses of nitrate and nitrite required to give 60 per cent. conversion of haemoglobin to methaemoglobin are—

Sodium nitrate (added to rumen)	420 mg per kg
Sodium nitrite (added to rumen)	170 mg per kg
Sodium nitrite (intravenously)	34 mg per kg

Oltmann and Crandall[26] find that the lethal dose of sodium nitrite for rabbits is 80 to 90 mg per kg. Winks, Sutherland and Salisbury[27] report that the lethal dose of sodium nitrite for pigs is 90 mg per kg, and that doses of 50 to 80 mg per kg give toxic symptoms.

The lethal doses of sodium nitrite, calculated as nitrogen, quoted above for adults and various animals can be summarised as shown below—

(1)	For 10-stone adult	33 mg per kg[4]
(2)	For 10-stone adult	22 mg per kg[12]
(3)	Rabbits	16 to 18 mg per kg[26]
(4)	Pigs	18 mg per kg[27]
(5)	Sheep	34 mg per kg[25]
(6)	Dogs	6·4 mg per kg[20]

A summary of the reported lethal doses of nitrate, calculated as nitrogen, is shown below—

(1)	For 10-stone adult	34 to 68 mg per kg[4]
(2)	For 10-stone adult	18 mg per kg[5]
(3)	Cattle..	75 mg per kg[21]
(4)	Cattle..	140 mg per kg[23]
(5)	Sheep	70 mg per kg[25]

NITRITES AND NITRATES IN WATER SUPPLIES—

In most natural waters, nitrate is the final product of the aerobic decomposition of nitrogenous organic matter. Its presence in a water supply is usually interpreted as an indication of contamination by organic matter, although this may have happened in the remote past and may not necessarily be serious. Nitrite indicates less complete oxidation and may show more recent and possibly dangerous contamination. Traces of nitrate are present in most natural waters, and it is only rarely that the concentration rises to a dangerous level. The recorded cases of poisoning caused by high concentrations in water supplies come under two headings—

(*a*) infantile cyanosis and
(*b*) animal poisoning.

(*a*) INFANTILE CYANOSIS—

Comly[7] cites from personal experience a number of cases of infantile cyanosis where symptoms were observed after ingestion of feeds made from waters containing 64 to 140 p.p.m. of nitrate-nitrogen. He quotes a number of other cases including one fatality.

Taylor[28] records a large number of cases of infantile cyanosis caused by nitrates in feed water.[29,30,31,32,33,34] Betke and Kleihauer[35] also refer to a case of infantile cyanosis occurring under similar circumstances. The reasons that make artificially fed infants particularly susceptible appear to be—

(*i*) the normal gastric achlorhydria of infants, already referred to,[1] and
(*ii*) the high relative fluid intake of an infant compared with that of an adult.[3]

In England, a baby under 15 lb requires about 2½ fluid oz. of water per pound body weight per diem.[3] In the tropics, this figure is much higher and may rise to about 10 fluid oz. per pound body weight per diem.[36] In comparison, a normal adult in England requires about 1·8 pints of liquid a day.[37] For a 10-stone man, this is equivalent to ¼ fluid oz. per pound body weight per diem. In the tropics, the requirement would be about 1⅓ fluid oz. per pound body weight per diem.

Waring[38] suggests that waters used for preparing infant feeds should not contain more than 20 p.p.m. of nitrate-nitrogen.

Ewing and Mayon-White[33] concur with the suggestion. The Committee of the Division of Medical Science of the United States National Research Council[39] regard any well water containing more than 10 p.p.m. of nitrate-nitrogen as unfit for consumption by infants. Wood[3] regards supplies containing less than 20 p.p.m. of nitrate-nitrogen as satisfactory for infant feeding, those containing 20 to 39 p.p.m. as undesirable, those containing 40 to 79 p.p.m. as unsafe and those containing more than 80 p.p.m. as dangerous. Any apparent discrepancy between these authors' findings may be explained by possible differences in climate.

(*b*) ANIMAL POISONING—

Cattle—Fincher[40] reports the death of 4 cattle after drinking from a supply containing 1000 p.p.m. of nitrate-nitrogen. Successive Sudan Government Analysts have recorded

deaths of cattle and camels after drinking from water supplies containing a high nitrate concentration.[41,42,43,44,45,46] In the majority of cases, the nitrate-nitrogen concentration was found to lie in the range 70 to 150 p.p.m., although three supplies containing as much as 870 p.p.m. have been recorded.[45,46] In a recent case, a supply found to contain 320 p.p.m. was fatal to cattle in about 1½ hours after watering.[45] Unfortunately, as far as the records show, the bodies of poisoned animals have not been examined by veterinary inspectors because the supplies have come from wells in remote areas, and any evidence has been lost before a post-mortem examination could be made. On the basis of available evidence, I introduced a tentative limit of 50 p.p.m. of nitrate-nitrogen, which has been applied in recent years in Khartoum when making recommendations on the suitability of a particular supply. However, Ibrahim has pointed out[45] that the nitrate concentration of a water supply may fluctuate within a wide range, and he re-examines regularly any supply with a nitrate-nitrogen concentration in the range 30 to 50 p.p.m. Willimott[47] has also had experience of cattle poisoning in Cyprus caused by well waters containing high concentrations of nitrate.

Pigs—Winks, Sutherland and Salisbury[27] record heavy mortality among pigs fed on a soup made by cooking beef and offal in a well water containing 290 to 495 p.p.m. of nitrate-nitrogen and less than 1·5 p.p.m. of nitrite-nitrogen. The soup itself contained 80 to 285 p.p.m. of nitrite-nitrogen.

Fish—Sanborn[48] states that water containing 2000 p.p.m. of sodium nitrate (330 p.p.m. of nitrate-nitrogen) is fatal to fish, whereas a concentration of 1000 p.p.m. of sodium nitrate (165 p.p.m. of nitrate-nitrogen) is without apparent effect.

Discussion and conclusions

In deducing a suitable limit for nitrates in water to be consumed by infants, adults and animals, the following assumptions have been made—

(*i*) that the nitrate is completely reduced to, and absorbed as, nitrite;
(*ii*) that the lethal dose of nitrite-nitrogen for men and cattle is about 20 mg per kg;
(*iii*) that one fifth of the lethal dose, *i.e.*, 4 mg per kg, is the maximum daily amount of nitrite-nitrogen that can be tolerated without giving rise to toxic symptoms.

When computing limits for cattle in tropical climates it should be remembered that in many places cattle are watered only once or twice a day, and that, as a consequence, large volumes are taken at a time.

On the above basis the limits shown in Table I for the permissible concentrations of nitrate-nitrogen in drinking water for infants, adults and cattle in England and in the tropics have been calculated.

Table I

Permissible limits for nitrate-nitrogen in drinking water

Consumer	Assumed weight, kg	Region	Maximum permissible dose of nitrate-nitrogen, mg	Approximate daily water intake, litres	Permissible limit of nitrate-nitrogen in water, p.p.m.
Infant ..	3	England	12	0·5	24
Infant ..	3	Tropics	12	2	6
Adult ..	60	England	240	1	240
Adult ..	60	Tropics	240	5·3	45
Cow ..	500	Tropics	2000	45	45

These figures can only be approximate, but they give general support to the limits already suggested. They emphasise the variations caused by differences in climate. They also show how very susceptible an infant artificially reared in the tropics can be to relatively small amounts of nitrates in drinking water. In areas where the water supplies have a high nitrate concentration special arrangements must be made for supplying infants with low-nitrate water. This is already being done in Norfolk and East Suffolk.[3]

It may also be concluded that, since the lethal doses of nitrate-nitrogen and nitrite-nitrogen for adults are not very different, most of the nitrate consumed is reduced to nitrite and absorbed into the blood stream. Thus it would appear that the significant factor that makes infants susceptible to nitrates is the high relative fluid intake and not the gastric achlorhydria.

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Some Aspects of Pipette Usage

Part I. Precision

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The method used in the manipulation of pipettes is described. The component errors in the variability of the volume delivered are analysed and their magnitudes determined or estimated. The resultant of these is shown to be in good agreement with the experimentally determined precision of the volume delivered.

The experiments described originated in an attempt to evaluate the errors in measuring volumes in a series of comparative titrations. They are not an attempt to show how precise the deliveries from a pipette can be made. This has been done by Bishop[1]; by observing the meniscus with a travelling microscope, by working under conditions in which the ambient temperature and pressure were remarkably uniform, by rigidly standardising every operation involved and by taking the values for which replication was satisfactory, he obtained standard deviations of 0·0002 to 0·0003 ml in the deliveries from 5- to 100-ml pipettes. Although this probably represents the maximum precision attainable with pipettes, it was not intended to indicate the precision obtainable in normal use.

However, we found that the precision obtainable under ordinary conditions was remarkably good and were prompted to investigate not only the various contributions to the over-all precision (Part I), but also the effect of changing certain variables in technique and in conditions (Part II).

General procedure—

The pipettes used were selected grade-B one-mark bulb pipettes and 2- and 50-ml pipettes (designated M2- and M50-ml hereafter) modified by collapsing the stem at the mark to form a capillary of about 1 mm inside diameter and about 1 cm long and making a new mark around the centre of this portion; the tip was drawn out to form a slender tip with thin glass walls. These modifications were designed to reduce errors associated with the tip and in setting to the mark.

All apparatus was cleaned before use with chromic - nitric acid cleaning mixture; it was then thoroughly rinsed with distilled water and finally with the liquid to be used for calibration. To avoid heating the working portion, the pipettes were held by the extreme top and by a 3-cm cube of plastic foam placed around the lower stem. The temperature recorded was that of the liquid in the reservoir (usually a 1-litre beaker nearly full) from which the pipette was being filled, unless (as in some special experiments) the temperature of the liquid differed markedly from that of the air, when the temperature of the liquid delivered was determined directly.

The pipettes were filled by suction until the meniscus was about 2 cm above the mark and the tip was wiped with a piece of absorbent tissue. With the pipette held vertically and its tip in contact with the wetted lip of a clean beaker, the meniscus was allowed to fall slowly until it was on the mark. The beaker was then removed, the meniscus level was checked, and the pipette was allowed to deliver freely (*i.e.*, with the tip in air) in a vertical position, the liquid delivered being collected in a 50-ml conical flask, which was closed with a rubber bung during weighing. When flow had ceased, the pipette was allowed to drain in the vertical position for 20 seconds, after which the tip was touched against the surface of the liquid and then withdrawn. The last process occupied about 2 seconds, hence the total drainage-time is quoted as 22 seconds. The modified pipettes were emptied as described above; after 20 seconds' drainage, the liquid in the tip was expelled with the tip in contact with the wall of the flask.

For finger control of the meniscus the slightly moistened forefinger was used. For "pneumatic control," a length of rubber tubing was fitted to the pipette and two screw-clips were placed 1 and 3 cm from the junction; with both clips fully open the pipette was filled to above the mark, the outer clip was closed and the final adjustment was made by manipulating the inner clip. The weights of liquid delivered were converted into volumes-at-20° C

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by means of the Tables given by Strouts, Gilfillan and Wilson.[2] The distilled water used was not boiled out—although boiled-out water is recommended by many authorities it is difficult to see how such water can be used without it partially reverting to its original state. Weighings were made with calibrated weights, and were recorded to 0·1 mg.

PRESENTATION OF RESULTS—

Volumes are calculated generally to 0·0001 ml, whether this is significant or not. Reproducibility is expressed as a standard deviation and is followed (in parentheses) by the number of determinations involved. The volume delivered, corrected to 20° C, is designated volume D 20° C in the Tables.

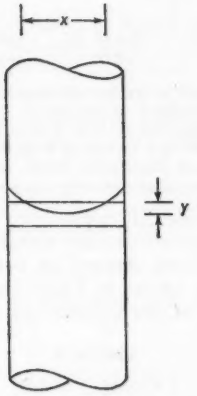

Fig. 1. Meniscus at a graduation mark

PRECISION OF SETTING TO THE MARK—

If a meniscus in perfect tangential contact with the top of a graduation line is allowed to move beyond this position, a region of mutual overlap is formed. If the width of this region is x (see Fig. 1), then the meniscus movement, y, corresponding to an increase of x from $x = 0$ to $x = x$, is given by—

$$y = x^2/4D_m \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where D_m is the apparent diameter of the meniscus. For large values of x and y, or when overlap occurs over more than the central portion of the meniscus, *i.e.*, where D_m is no longer sufficiently uniform, the value of y given by equation (1) is too small. Such errors do not normally arise until the meniscus has appeared below the bottom of the graduation.

Values of D_m were determined for water in ordinary glass tubing of various internal diameters (D_t). For $D_t \leqslant 0{\cdot}6$ cm (which normally includes all sizes of pipette up to 50 ml) a linear relationship was observed—

$$D_m = 1{\cdot}80\ D_t \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

As D_t increased above 0·6 cm, D_m increased rapidly until at $D_t = 1{\cdot}2$ cm it was about twice as large as the value given by equation (2).

Despite their subjective nature, three kinds of setting to the mark may be distinguished *in terms of the value of* x—

(1) Meniscus just on the graduation line ($x = x_1$).
This is obtainable only under the best conditions with "pneumatic control" of the meniscus level. A position where the meniscus was perfectly tangential to the top of the graduation was never observed; there was always a region where contact was uncertain.

(2) Meniscus moderately on the graduation line ($x = x_2$).
This represents careful working with finger control of the meniscus level.

(3) Meniscus generously on the graduation line, which is usually equivalent to very slightly below it ($x = x_3$).

This represents routine working. Provided that the meniscus is viewed against a suitable background this setting should be the extreme case.

If D_t is very small, the meniscus cannot be distinguished accurately without a lens, whereas if D_t is too large, the value of x depends too much on individual marks.

For values of D_t between 0·1 and 0·6 cm, the following relationships were observed—

$$x_1 \simeq 0{\cdot}33\ D_t;\ x_2 \simeq 0{\cdot}51\ D_t;\ x_3 \simeq 0{\cdot}64\ D_t \quad .. \quad .. \quad .. \quad (3)$$

From equations (1), (2) and (3), y and the corresponding volume (ΔV) between $y = 0$ and $y = y$ can be expressed in terms of D_t—

Setting	1	2	3*
y, cm	$0{\cdot}015\ D_t$	$0{\cdot}036\ D_t$	$0{\cdot}057\ D_t$
ΔV, ml	$0{\cdot}012\ D_t^3$	$0{\cdot}028\ D_t^3$	$0{\cdot}044\ D_t^3$

* For setting 3, the calculated values of y are usually greater than the thickness of the graduation line and hence are in agreement with the definition of setting 3. However, a definition based entirely upon an emergence of the meniscus below the graduation line is unsatisfactory, because (*a*) graduation lines vary in thickness, (*b*) it is difficult to tell when the meniscus begins to appear below the line and (*c*) there is considerable refraction around the edges of the mark, which makes the apparent thickness of the mark, *as measured by meniscus movement*, greater than the true thickness.

Other things being equal, the volume ΔV represents the range within which the volume contained by the pipette at the given setting should lie. The corresponding standard deviation (S_1) of this contained volume cannot be calculated accurately, but is likely to be about $\Delta V/2$ (see Conway[3]). The values in Table I are typical and are used later in the calculation of the over-all precision of the volume delivered.

TABLE I

TYPICAL VALUES

Pipette volume, ml	Setting	D_t, cm	ΔV, ml	S_1, ml
M2	$x < 0{\cdot}2$ mm	0·1	0·0004	0·0002
5	3	0·37	0·0023	0·0012
10	2	0·36	0·0013	0·0006
10	1	0·36	0·0006	0·0003
20	1	0·47	0·0013	0·0006
50	1	0·62	0·0029	0·0015
M50	$x < 0{\cdot}2$ mm	0·10	0·0004	0·0002

TABLE II

RESULTS FOR GRADE-B BURETTES

Burette volume, ml	Setting	Delivery time, seconds	D_t, cm	ΔV, ml	$\Delta V/2$, ml	$S_t/\sqrt{2}$, ml	Number of duplicates
5	2	210	0·326	0·00097	0·0005	0·0004	17
50	–*	300	1·11	0·0082	0·0041	0·0030	15

* This was slightly better than setting 2. Because D_t is large, the value of ΔV was calculated from the observed values of x, D_m and D_t.

For burettes with suitably long delivery times, errors other than those in reading the position of the meniscus are negligible, and a direct comparison between the calculated and observed precision is immediately possible. The results in Table II were obtained with grade-B burettes fitted with a reading device to avoid parallax and with all readings made at a mark; the estimate of the standard deviation in reading the burette is $\Delta V/2$ as before, and this should be equal to $1/\sqrt{2}$ times the observed precision (S_t) of the volume delivered.

The observed values of S_t were found to be independent of the volume delivered. The agreement between $\Delta V/2$ and $S_t/\sqrt{2}$ is remarkably good in view of the approximations involved.

Manipulation of pipettes when setting to the mark—It is customary to adjust the meniscus to the mark with the tip in contact either with the liquid surface or with a wetted surface. When contact is broken there is an inevitable change in the associated surface-tension forces,

and this, by virtue of the elasticity of the air enclosed between the mark and the end of the pipette, causes the liquid to retract slightly. The most reproducible conditions are obtained by holding the tip against a clean wetted surface at a large enclosed angle. If the surface is neither clean nor wet the error can be appreciable, *e.g.*—

Volume delivered by 10-ml pipette at 20° C—
Tip held against clean wetted surface—9·9973 ± 0·0008 ml (12)
Tip held against dirty surface—9·9996 ± 0·0019 ml (5).

The elasticity of the enclosed air also requires that the filled pipette be handled gently to avoid expulsion of the liquid by jolting; similarly, the tip should never be wiped with tissue at this stage (compare Conway[3]) otherwise an appreciable volume of liquid may be removed.

ERRORS ASSOCIATED WITH THE TIP—

Owing to capillary forces a small volume of liquid (the tip-volume) is always left in the tip of the pipette after delivery has ceased. This is of no consequence provided that the tip-volume remains constant from delivery to delivery.

At equilibrium, the surface-tension forces inside the tip are balanced by the surface-tension forces outside the tip *plus* the hydrostatic force of the liquid column inside the tip. If the attitude of the tip and the density and surface tension of the liquid are constant, then the reproducibility of the tip-volume is determined solely by the reproducibility of the surface-tension forces outside the tip, *i.e.*, those forces arising from the curvature of the liquid surface around the outside of the tip. This curvature is markedly affected by the mode of contact between the tip and the liquid; thus it is dependent upon whether the tip is held on the bulk liquid surface or on a wetted surface, and, if the latter, upon what angle the tip makes with the surface.

TABLE III

EFFECT OF CHANGE IN SURFACE TENSION ON TIP-VOLUME

Pipette volume, ml	10	20	50
Tip-volume with water (γ = 73 dynes per cm), ml	0·0133 ±0·0003(4)	0·0203 ±0·0008(4)	0·0178 ±0·0005(7)
Tip-volume with Teepol solution (γ = 30 dynes per cm), ml	0·0070 ±0·0005(3)	0·0090 ±0·0006(3)	0·0075 ±0·0008(8)

The effect in terms of a change in tip-volume is shown by experiments with the 10-ml pipette held vertically. The differences between the tip-volume (determined by weighing the liquid expelled) obtained after withdrawing the tip from a position just touching the liquid surface and that after withdrawing the tip from different positions were—

Touching wetted glass wall, enclosed angle 60° .. +0·0015 ml
Touching wetted glass wall, enclosed angle nearly 0° +0·003 ml
Immersed 1 mm in the liquid, hurried withdrawal .. −0·003 ml

(Total tip-volume: 0·013 ml; internal diameter of tip at meniscus: 2 mm, at end of tip: 0·7 mm.)

The effect of a change in surface tension (γ) on the tip-volume (obtained after withdrawing the vertically held pipette from a position just touching the liquid surface) together with typical values for the tip-volume are shown in Table III. The reproducibility of the tip-volume is seen to be independent of tip-volume, the average value being ±0·0005; this has to be corrected for the reproducibility of the minute volume of liquid left after expulsion (but which cannot be determined accurately) and the corrected reproducibility is estimated to be about ±0·0004 or less. The error caused by variability of the tip-volume is thus much less than the possible variation in the tip-volume itself.

Although a reduction of the internal diameter at the tip serves to diminish some of the errors so far considered, in principle the only way to eliminate them is to expel the tip-volume and include it in the volume delivered. Most authorities (without apparent justification) condemn such a procedure; admittedly it is impossible in practice to expel all the liquid in the tip, and with the above pipettes the amount of liquid remaining was about 0·0004 ml, although up to 0·0008 ml was occasionally observed. Even so, this is very small. Complete expulsion is hindered by the large amount of glass at the tip (as indicated in a British Standard[4]); if the tip is drawn out to leave a minimum of glass at the tip, as in the modified pipettes, the volume remaining after expulsion can be reduced to about 0·0002 ml.

Considering the effect of surface tension alone on the tip-volume, as intrinsic changes greater than 2 dynes per cm are unlikely with dilute aqueous solutions, there is no need to expel the drop, provided that the treatment of the tip at the end of the delivery is rigidly standardised. If this is not done, or cannot always be done, a procedure involving expulsion of the tip-volume may be the best for varied use.

VARIATION OF THE DELIVERY TIME AND ASSOCIATED ERRORS—

Owing to the slowness of flow at the end of the delivery it is difficult to determine with any precision the time at which flow stops. For this reason, deliveries were timed to a mark about 3 cm above the tip, the meniscus movement being sufficiently rapid at this mark to allow accurate timing. For vertically held pipettes the delivery times (in seconds), measured by a stop-watch reading to 0·1 second, are shown in Table IV.

TABLE IV

DELIVERY TIMES OF PIPETTES

Temperature: 16° C

	10-ml pipette		50-ml pipette	
	Full delivery, seconds	Lower stem, seconds	Full delivery, seconds	Lower stem, seconds
Free delivery	18·65 ± 0·07(6)	4·60 ± 0·00(3)	41·63 ± 0·07(11)	3·30 ± 0·00(3)
Tip immersed 0·5 cm in water	19·10 ± 0·07(6)	4·60 ± 0·05(4)	42·35 ± 0·00(3)	3·35 ± 0·05(5)
Tip held against wetted surface	18·07 ± 0·07(6)	4·25 ± 0·05(5)	41·25 ± 0·05(3)	3·10 ± 0·10(6)

The differences in the delivery times caused by a change in the method of emptying the pipette are as expected. The differences between the times of delivery from the lower stem are smaller than those for the whole delivery, indicating that a change of conditions affects the whole delivery rather than that from the lower stem alone.

A change in delivery time causes a change in the volume of liquid remaining on the walls after delivery (the wall-film). As shown by Conway[3] and confirmed by us (see Part II), the wall-film is proportional to (delivery time)$^{-\frac{1}{2}}$; therefore, from the values for the wall-film given in Part II, a change of 0·5 second in the delivery time produces a change of 0·0006 ml in the wall-film (and consequently, by difference, in the volume delivered) of, fortuitously, either pipette, other things being equal. Determinations of the time for full delivery to the tip showed a precision of ±0·15 second, which is a combination of personal error and a discontinuous time distribution; the real variation is less than ±0·1 second (compare Conway[3]), which, with constant delivery technique, corresponds to a precision for the wall-film of better than ±0·0001 ml.

TABLE V

OVER-ALL PRECISION OF VOLUME DELIVERED

Pipette volume, ml	M2*	5	10		20	50	M50	Remarks
Setting No.†	$x<0·2$ mm	3	2	1	1	1	$x<0·2$ mm	—
Delivery time, seconds ..	22	15	22	22	31	44	41	—
S_1	0·0002	0·0012	0·0006	0·0003	0·0006	0·0015	0·0002	—
S_2	0·0000	—	0·0001	0·0001	0·0002	0·0004	0·0004	≡ ± 0·05° C
S_3	0·0002	—	0·0004	0·0004	0·0004	0·0004	0·0002	—
S_4	0·0000	—	0·0001	0·0001	0·0001	0·0001	0·0001	—
S_5	0·0000	—	0·0002	0·0002	0·0002	0·0002	0·0002	≡ ± 2 seconds (See Part II)
S_t (calculated) ..	0·0003	0·0014	0·0008	0·00056	0·0008	0·0016	0·0006	—
S_t (observed) ..	0·00028	0·0016	0·0008	0·00049	0·0008	0·0016	0·0006	Pipette clamped vertically
No. of determinations	11	10	17 + 12	15	9	12	50	—
S_t as percentage of total delivery	0·02	0·03	0·008	0·005	0·004	0·003	0·001	—

* Drainage time, 5 seconds. † See p. 435.

Unfortunately, a change in the wall-film due to slight vibrations imparted by the hand (see Part II) is not reflected by a corresponding change in the delivery time—

	Delivery time, seconds
50-ml pipette, clamped vertically	40·90 ± 0·00 (4)
50-ml pipette, hand-held vertically	40·90 ± 0·05 (3)

(The difference between the above and previous values for the same pipette is due to the use of a different mark.)

Further, there are other factors (*e.g.*, surface tension and viscosity—see Part II) that can affect the wall-film with but little effect upon the delivery time. Consequently, any conclusions regarding the wall-film that are based upon delivery times must be made under conditions in which such influences are excluded.

The direct determination of the reproducibility of the wall-film is subject to so many errors that it was not attempted.

OVER-ALL PRECISION OF THE VOLUME DELIVERED—

This will be the resultant of the component reproducibilities (S_n, standard deviations expressed as a volume), *i.e.*—

Setting to the mark	S_1	Precision of the total volume *before* delivery.
Temperature uncertainty	S_2	
Tip-volume	S_3	Precision of the residual volume *after* delivery.
Wall-film	S_4	
Drainage	S_5	

As all these components are independent, the over-all variance (S_t^2) will be the sum of the individual variances, *i.e.*, $S_t^2 = \Sigma S_n^2$. The individual variances have been measured or estimated for a number of pipettes, and the results, together with the observed values for the over-all reproducibility, are given in Table V.

The agreement between the observed and calculated values of S_t is remarkably good and confirms the general correctness of the appraisal of the component errors. The largest error is in setting the meniscus to the mark. However, incorrect or unsuitable technique can increase the other errors by a factor of ten or more (see Part II), whereas the value of S_1 would remain sensibly constant.

The experimental values for S_t are in agreement with the best of the somewhat fragmentary data available in the literature.[5,6,7] They are significantly better than those found by Conway,[3] and this must be ascribed to the technique he employed.

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Some Aspects of Pipette Usage

Part II. The Effects of Certain Variables

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Investigations have been made of the effects of surface tension, viscosity, the temperature rise and the vibrations caused by holding the pipette by hand, inclination of the pipette, thermal hysteresis and grease on the volume delivered by the pipette and on other relevant properties of the pipette. Conway's relationship between the delivery time and the wall-film is confirmed, and his work on the effect of temperature on the wall-film is corrected and extended. The most serious sources of variable error are summarised and recommendations are made whereby these can be minimised without undue inconvenience.

The variables considered can be divided into two groups: (*a*) those over which little or no control is possible, *e.g.*, changes in surface tension, and (*b*) those that can be controlled, most of which are variations in technique. Investigations of the first group have been made to discover their modes of action and the magnitudes of their effects upon the volume delivered. Investigations of the second group are similar, but, in addition, conditions have been determined in which their effects on the volume delivered are minimised.

Influence of surface tension—

Deliveries were made from vertically clamped pipettes filled with water ($\gamma = 73$ dynes per cm) or with a 1 per cent. v/v solution of Teepol ($\gamma = 30 \pm 1{\cdot}5$ dynes per cm; relative density $1{\cdot}000932 \pm 0{\cdot}000017$ (5 + 5)). The results are shown in Table I.

Table I

Effect of surface tension

		10-ml pipette	20-ml pipette	M50-ml pipette
Volume, D 20° C	water, ml	9·9973 ± 0·0008(17)	20·0221 ± 0·0008(9)	48·4950 ± 0·0006(5)
	Teepol, ml	9·9841 ± 0·0002(5)	20·0133 ± 0·0007(4)	48·4563 ± 0·0008(4)
Difference, ml		0·0132	0·0088	0·0387
Volume of wall-film*	water, ml	0·050	0·063	0·084
	Teepol, ml	0·067	0·084	0·121

*Obtained by subtracting the volume delivered *plus* the tip-volume from the total capacity and correcting to 20° C (see later).

The presence of Teepol increases the wall-film by about 40 per cent., and, although this increase is partly compensated by the reduction in the tip-volume (see Part I), the volume delivered is less. Decrease of surface tension also decreases the meniscus volume[1]; for a tube of diameter 0·5 cm, a change in surface tension from 73 to 40 dynes per cm reduces this volume by 0·001 ml. The change in meniscus volume decreases rapidly as the diameter of the tube is decreased; hence the effect here is small.

The presence of Teepol also affects the delivery time; for the 10- and 50-ml pipettes the delivery times were decreased by about 0·5 second. Such a change corresponds to an increase of about 0·0006 ml in the wall-film of, fortuitously, either pipette. Alternatively, if the decrease in delivery time is attributed to a decrease in the viscosity (see later), this corresponds to a decrease of about 0·006 ml in the wall-film. Clearly, the major change in the wall-film is not caused by a change in the delivery time.

If the change in the wall-film with surface tension is linear, then a change of 1 dyne per cm in surface tension produces a change of about 1 per cent. in the wall-film; as the differences in surface tension encountered with dilute aqueous solutions are usually not greater than 2 dynes per cm, the errors here are not serious. Errors due to the decrease in surface tension by contamination may be more significant, but these can usually be avoided by taking suitable precautions.[1]

Whatever the conditions, a small wall-film is clearly advantageous.

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INFLUENCE OF VISCOSITY—

A change in viscosity may result from the presence of some substance in solution or from a change in temperature; the former is considered below and the latter is considered on p. 445.

The viscosity of water was increased by the addition of a boiled and filtered solution of a cellulose wallpaper-adhesive, chosen for its negligible effect on the surface tension and for the small concentration required to change the viscosity markedly. The resulting viscosity was determined by treating the modified 50-ml pipette as a Redwood viscometer, when the following equation is applicable—

$$\eta/d = At - B/t$$

where η is the viscosity in poises, d is the density of the solution in grams per millilitre, A and B are constants and t is the delivery time in seconds. The values of A (0·00097) and B (1·20) were determined by calibrating with water at different temperatures.

The effect of a change in viscosity on the properties of the M50-ml pipette is shown in Table II.

TABLE II

INFLUENCE OF VISCOSITY

Temperature: 18·5° C

		Solution			
	Water	1	2	3	4
Relative density	1·00000	1·00016	1·00035	1·00070	1·00135
Specific viscosity	1·00	1·5	2·1	3·2	5·1
Surface tension, dynes per cm	73	72	72	72	72
Delivery time, seconds ..	41	44	48	56	72
Drainage,* ml	0·001	0·001	0·001	0·002	0·002
Volume, D 20° C, ml ..	48·4958 ±0·0005(5)	48·4403 ±0·0007(4)	48·4011 ±0·0023(4)	48·3567 ±0·0009(4)	48·2957 ±0·0020(4)
Wall-film, ml	0·085	0·141	0·180	0·224	0·285

* Over the standard drainage time of 22 seconds.

An increase in the viscosity causes a decrease in the volume delivered. This cannot be the result of increased delivery time, because the effect is too large and in the wrong direction. If the volume of the wall-film is plotted against viscosity, a shallow curve passing through the origin is obtained. From this, a change of ±1 per cent. in the viscosity of water produces a change of about ±0·8 per cent. in the wall-film; such a change in viscosity is a typical value for 0·1 M solutions.

As with surface-tension effects, a small wall-film is advantageous.

EFFECTS ASSOCIATED WITH HOLDING THE PIPETTE BY HAND

THERMAL EFFECTS—

If the pipette is held by hand, appreciable heat may be imparted to it in a remarkaby short time. The results in Table III were obtained with a 10-ml pipette held at the extreme top, delivery being made on to the bulb of a thermometer. The figures quoted are the differences between the temperature (in ° C) recorded on the thermometer during the delivery and that of the water in the reservoir. (The difference in temperature between the air and the water in the reservoir never exceeded 1° C).

TABLE III

THERMAL EFFECT OF HOLDING 10-ml PIPETTE BY HAND

	Held by hand	Held by gloved hand
1. Held at extreme top	0·1	not detected
2. Bulb held through delivery	1·5	0·2
3. Lower stem held throughout delivery	0·7	0·2
4. Lower stem held for adjustment only	0·5	not detected
5. As 4, rapid adjustment	0·2	not detected
6. Lower stem held by plastic-foam cube throughout delivery ..	not detected	—

These differences are quite significant; a change of 0·5° C in the temperature of the water could in certain circumstances lead to an error of 0·001 ml, which is larger than the

observed precision of delivery for this pipette. Thus in precise work handling the pipette without adequate insulation should be avoided.

The conversion of kinetic energy into heat can affect the pipette only when the liquid is sucked into the pipette. The temperature rise can be shown to depend upon the speed of filling, but for normal pipettes is not likely to exceed 0·02° C.

VIBRATION EFFECTS—

These can be demonstrated with a 50-ml pipette and a strongly coloured solution; the thickness of the wall-film *in the bulb* (as indicated by the depth of colour) is greater when the pipette is held by hand during delivery than when it is clamped in position. This can only be caused by the slight vibrations imparted to the pipette when it is held by hand; these vibrations produce oscillation of the meniscus level, which simulates an apparent increase in the rate of descent and thus leads to a larger wall-film.

In the experiments (see Table IV) carried out to investigate this effect, the hand-held pipette was supported with one hand at the extreme top and the other at the foam insulator, both elbows being on the bench; this position was slightly less steady than when the pipette was held at the extreme top only and with the elbow on the bench. Absolute steadiness could not be obtained, even with the forearm resting on a solid support.

TABLE IV

THE EFFECT ON DELIVERY OF HOLDING THE PIPETTE BY HAND

Pipette volume, ml	Internal diameter of bulb, mm	Volume, D 20° C			Wall-film (with pipette clamped), ml
		Clamped, ml	Hand-held, ml	Difference, ml	
10	15	9·9973 ± 0·0008(17)	9·9973 ± 0·0008(12)	0·0000	0·050
20	20	20·0221 ± 0·0008(9)	20·0194 ± 0·0013(10)	0·0027	0·063
50	29	50·0641 ± 0·0016(12)	50·0493 ± 0·0034(16)	0·0148	0·108
M50	29	48·4950 ± 0·0006(5)	48·4804 ± 0·0045(6)	0·0146	0·084
		48·4563 ± 0·0008(4)*	48·4502 ± 0·0020(4)*	0·0061*	0·121*

* Results with Teepol solution; $\gamma = 30$ dynes per cm.

The effect of vibration is more pronounced as the capacity increases, and this is probably related to the increasing diameter of the bulb. The thickness of the wall-film in the bulb of the 10-ml pipette is greater than that of the 50-ml pipette (0·0023 and 0·0011 cm, respectively) and the thicker wall-film may accommodate the movement better. This view is corroborated by the decreased effect observed with a solution of Teepol; the wall-film is increased and the effect of vibration is more than halved. The effect here of surface tension could not be determined.

If the effect of vibration is indeed dependent on the thickness of the wall-film, then, other things being equal, it should increase as the delivery time is increased, thereby obviating any other advantages to be gained by this procedure.

The effect of extreme vibration on the volume delivered by the 10-ml pipette is shown in Table V.

TABLE V

EFFECT OF VIBRATION ON VOLUME DELIVERED FROM 10-ml PIPETTE

Condition	Volume, D 20° C, ml	Difference from volume in 1, ml
1. Clamped	9·9973 ± 0·0008(17)	—
2. Gentle vibration	9·9831 ± 0·0017(5)	0·0141
3. Vigorous shaking	9·9593 ± 0·0124(5)	0·0380

In agreement with Ponndorf[2] and Bishop,[3] but not Conway,[4] we find that manual holding can significantly affect the precision of the volume delivered, although the effect is small compared with the change in the volume delivered.

No increase in the wall-film was observed with the 5- and 50-ml burettes (Part I) even with gentle vibration or knocking.

INFLUENCE OF INCLINATION OF A PIPETTE ON THE DELIVERY AND DRAINAGE—

A change in the inclination of the pipette has three distinct effects; first, the delivery time is altered because of the change in the head of liquid, secondly, the drainage is affected and thirdly there is a change in the circumference of the meniscus over those parts of the

pipette, *e.g.*, the bulb, where capillary forces do not predominate. The net result of these changes depends largely upon the individual characteristics of the pipette.

If the wall-film is not too large and the pipette is vertical, drainage from the upper stem and the bulb travels only slowly down the pipette and does not reach the tip until the normal drainage time of 15 to 20 seconds has elapsed. Thus the only drainage from which errors may arise is that from the lower stem. If the wall-film is large, as after rapid deliveries, the rate of drainage is increased and the above is no longer true.

If the pipette is tilted, drainage occurs in two main sections down each side of the pipette. The liquid from these two sections collects along the lower wall of the pipette and a relatively large volume of liquid accumulates there. Most of this runs rapidly down the length of the pipette and reaches the tip usually before the normal drainage time of 15 to 20 seconds has expired. This will occur whether the pipette has been emptied in a tilted or in a vertical position.

This view is confirmed by the results shown in Table VI, obtained with the M50-ml pipette clamped in position; the drainage was measured by observing the liquid level in the calibrated tip.

TABLE VI

EFFECT OF INCLINATION ON DRAINAGE FROM M50-ml PIPETTE

Angle to vertical	0°	30°	60°
Delivery-time, seconds	41·5	47	60
Total drainage, 0 to 20 seconds, ml	0·001	0·009	0·017
Total drainage, 20 to 40 seconds, ml	0·013	0·016	0·014
Rate of drainage, ml per 20 seconds at 20 seconds	0·003	0·012	0·017

Provided that the minimum possible drainage time was used, a change in inclination produced little or no change in the volumes delivered by our pipettes. With drainage times of 20 seconds, an increase (ΔV) in volume delivered was observed due to increased drainage. The values of ΔV (see Table VII) agree well with the independently determined increases in drainage.

TABLE VII

VALUES OF ΔV

Pipette, ml	10	M50	
Angle to vertical	45°	30°	60°
ΔV 2 seconds' drainge, ml	0·000	—	—
ΔV 20 seconds' drainage, ml	0·009	0·012	0·015
Increase in total drainage, 0 to 20 seconds, ml	0·010	0·008	0·016

These results in Table VII suggest that the effect of the increase in delivery time obtained on tilting the pipette is a subsidiary effect and is compensated by the effect of the change in the circumstances of the meniscus, the exactness of the compensation depending probably on the geometry and the delivery time of the pipette.

It follows from the foregoing that no general conclusions can be made about the effect of inclination of the pipette. However, it is clear that, for consistent delivery and minimum drainage, the best position for the pipette is a vertical one—this is also the most well defined. It is also advantageous to use a short (a few seconds) drainage time, since the rate of drainage increases rapidly during the first minute; on the other hand, owing to the slowness of flow towards the end of the delivery, the drainage time must not be too short, otherwise errors may arise from a premature withdrawal of the pipette. It is interesting to note that the National Physical Laboratory proposes to alter its standard drainage time from 15 to 3 seconds, although, according to an initial report,[5] this is in the interests of speed rather than from considerations such as those described above. There is also a smaller absolute error in counting 3 seconds than in counting 15 seconds.

INFLUENCE OF DELIVERY TIME ON THE WALL-FILM—

Other things being equal, a longer delivery time (t) results in a smaller wall-film; according to Conway,[4] the wall-film is proportional to $t^{-\frac{1}{2}}$ both for pipettes and burettes. This relationship was confirmed as described below.

The delivery times of the 10-, 20- and M50-ml pipettes were varied by partly closing the top of the pipette with a rubber tube and a screw-clip.* The volumes of water and of Teepol solution ($\gamma = 30$ dynes per cm) delivered for different delivery times were determined with the pipettes clamped vertically and with standard drainage time (22 seconds).

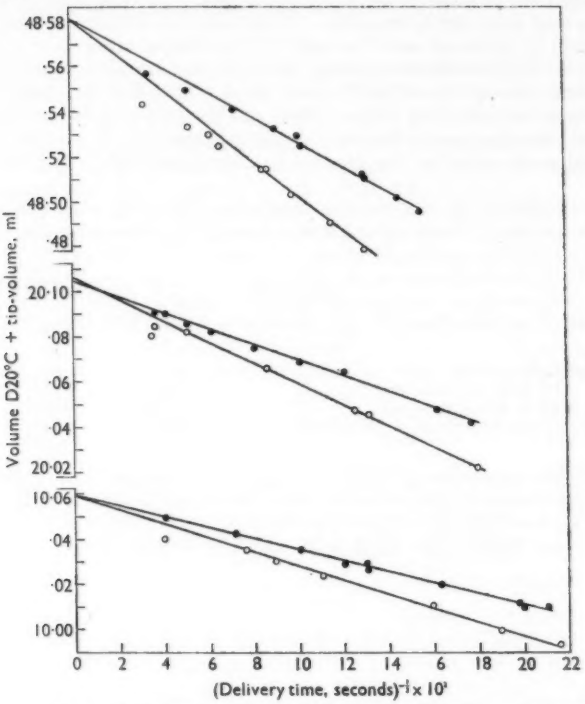

Fig. 1. Influence of delivery time on the wall-film; results for the 10-, 20- and M50-ml pipettes: ●, with water; ○, with Teepol solution

After the appropriate tip-volume had been added, these volumes were plotted against $t^{-\frac{1}{2}}$, when a linear relationship was obtained (see Fig. 1). The deviations that occur for $t > 5$ minutes are probably due to evaporation or to very slow final emptying (compare Stott[6]).

TABLE VIII

CALCULATED AND DETERMINED VALUES FOR WALL-FILM

Pipette, ml		10	20	50	M50
Total capacity	water, ml	10·060	20·105	—	48·581
	Teepol, ml	10·059	20·106	—	48·581
Wall-film,* ml		0·050	0·063	—	0·084
Wall-film by direct determination, ml		0·051	0·063	0·108	0·082

* Calculated by subtracting the values for the volume delivered and the tip-volume from the total capacity.

If the lines are extrapolated to $t^{-\frac{1}{2}} = 0$, the total capacities of the pipettes can be obtained with reasonable accuracy. By subtracting from these values the volume delivered under normal conditions and the tip-volume, the wall-film for normal delivery conditions can be obtained. Some results are shown in Table VIII. The values for the wall-film are corrected

* This is preferable to the method used by Stott,[6] *i.e.*, grinding off small portions of the tip. His experiments suggest that, with the same pipette, the two methods give different results, but when his results are re-plotted as a relationship between volume delivered and $t^{-\frac{1}{2}}$, parallel straight lines are obtained. If these are extrapolated to $t^{-\frac{1}{2}} = 0$, different total capacities are indicated. This is probably caused by a change in the tip-volume, which was not determined.

to 20° C (see later). The direct determinations were made by filling the pipette to the mark with 0·02 M potassium permanganate, care being taken not to rinse above the mark. The pipette was clamped vertically, and emptied and drained in the standard manner; the tip-volume was then expelled. The liquid remaining in the pipette was rinsed into a 50-ml calibrated flask, the solution diluted to the mark, and the permanganate determined absorptiometrically.

The total capacity obtained is the same whether water or Teepol solution is used and the calculated values for the wall-film agree with those determined directly. The results therefore confirm the linear relationship between the volume of the wall-film and $t^{-\frac{1}{2}}$ for pipettes clamped in a vertical position. For hand-held pipettes, a similar relationship applies.[4]

In this report, all corrections to volumes delivered for changes in delivery time have been calculated on this basis.

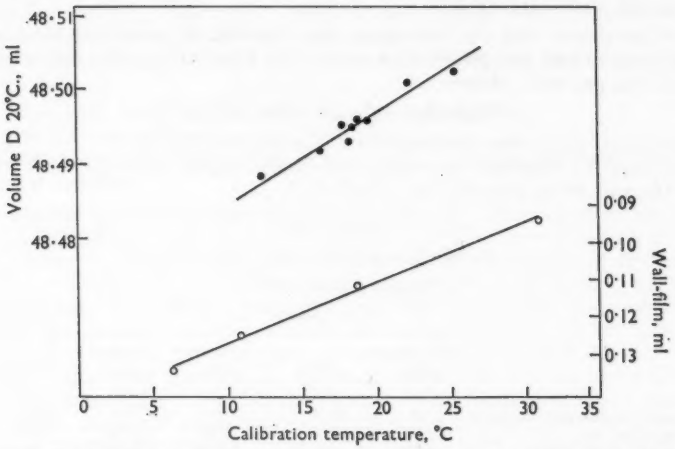

Fig. 2. Variation of wall-film with temperature: ●, present work with M50-ml pipette; ○, Conway[4] with 25-ml pipette

INFLUENCE OF TEMPERATURE OTHER THAN EXPANSION EFFECTS—

The corrections for temperature changes normally applied are those incorporated in tables for the effect of temperature on the density of water, on the capacity of the glass vessel and on the density of air (calibration only). As both viscosity and surface tension are temperature-dependent, the wall-film should be affected by changes in these quantities with temperature. Conway[4] has determined the effect for a 25-ml pipette (see Fig. 2) and his results are discussed subsequently.

The effect was measured by determining the volume delivered by the M50-ml pipette (clamped vertically) at various temperatures. These volumes were then corrected to 20° C in the normal way. When these volumes are plotted against the temperature of calibration, they lie on a straight line of slope 0·0012 ml per °C (see Fig. 2).

This change can be related to the wall-film as shown below—

	This work	Conway
Change in wall-film, ml per °C ..	−0·0012	−0·0016
Wall-film at 20° C, ml..	0·084	0·110
Change of wall-film, % per °C ..	−1·4 ± 0·1	−1·45 ± 0·1

The figure given by Conway,[4] and quoted by Sutton,[7] for this variation with temperature is 0·006 per cent. of the whole delivery for an increase of 1° C. This figure is of little value, as it applies only to the particular pipette that Conway used. The change must be related to the wall-film, and the figure −1·4 per cent. of the wall-film per °C is of general applicability.

The observed effect of temperature on the volume delivered at 20° C by the M50-ml

pipette can be compared with that calculated from the separately estimated changes due to viscosity and surface tension. If the temperature is increased from 15° to 25° C—

Observed increase in volume delivered	0·012 ml
Correction for decrease in delivery time (1·5 seconds)	+0·002 ml
Correction for decrease in surface tension (1·5 dynes per cm)	+0·002 ml
Corrected increase, which should be due to viscosity change alone ..	0·016 ml

The decrease in viscosity is 25 per cent. referred to that at 20° C. Hence the decrease in the wall-film, from p. 441, is 0·017 ml. The agreement is satisfactory. These calculations show that the main contribution to this influence of temperature is the effect of a change in the viscosity. As the observed effect is directly proportional to the wall-film, a small wall-film is again advantageous. For accurate work, the effect is sufficiently large to require an additional correction to be made.

DISTRIBUTION OF THE WALL-FILM—

Conway[4] has shown that the volume of the wall-film of a vertical tube can be related to the diameter (d in cm) and length (l in cm) of the tube, and to the rate of descent of the meniscus (v in cm per sec.), thus—

$$\text{Wall-film, ml} = 0{\cdot}00345\ d^{1{\cdot}5}\ lv^{0{\cdot}5}$$

(the temperature at which this equation is valid is not stated).

This was used to calculate the wall-films in the upper stem, the bulb and the lower stem of the 10- and 50-ml pipettes (see Table IX).

TABLE IX

WALL-FILM DISTRIBUTION

Temperature: 16° C

	10-ml pipette			50-ml pipette		
	Upper stem	Bulb	Lower stem	Upper stem	Bulb	Lower stem
Length,* cm	5·0	5·5	17·5	10·0	8·5	18·0
Time of meniscus descent, seconds	0·9	13·3	7·0	2·1	36·6	5·0
Internal diameter, cm	0·36	1·4	0·36	0·62	2·8	0·43
Wall-film (calculated), ml ..	0·018	0·018	0·018	0·036	0·044	0·033
Percentage of total wall-film ..	33	33	33	32	39	30
Total wall-film (calculated), ml ..		0·055			0·113	
Total wall-film (measured), corrected to 16° C, ml		0·053			0·114	

* Corrected for the change in diameter at the shoulders.

Despite the errors of measurement and the absence of a reference temperature, the agreement between the calculated and measured values for the total wall-film may be taken as confirmation of the essential correctness of Conway's equation.

The results in Table IX show that the wall-film is about equally distributed between the three portions of the pipette. Useful reductions in the wall-film, and hence increases in accuracy, can therefore be obtained by reducing the dimensions of the stems as much as possible. Thus for our 50-ml pipette, if the upper stem were reduced to zero length and the lower stem from 18 to 10 cm in length and from 0·43 to 0·20 cm in diameter, the wall-films on these portions would be reduced to nil and 0·012 ml, respectively, and the total wall-film from 0·113 to 0·057 ml. If, in addition, the delivery time were increased from 44 to 60 seconds, the wall-film would be further reduced to 0·049 ml, *i.e.*, an over-all reduction of about 60 per cent. The errors in the volume delivered due to changes in viscosity, surface tension and temperature would be correspondingly reduced.

INFLUENCE OF THERMAL HYSTERESIS—

The thermal hysteresis of volumetric glassware is usually assumed to be fairly large. Kolthoff and Stenger[9] state that the effect is much smaller than has been previously supposed, but quote no data. After heating the M50-ml pipette (made from "soda" glass) to 100° C for 1 hour, an increase of 0·003 ml in the volume delivered was observed and was unchanged after 4 days. The effect here is small.

INFLUENCE OF GREASE—

Conflicting statements appear in the literature about the influence of grease; its presence has been said to increase,[9] decrease[10] and both to increase and decrease[11] the volume delivered. Our own experiments with the M50-ml pipette were inconclusive, except that in the presence of grease a large decrease in precision was observed.

CONCLUSIONS

The results of the investigation are in agreement with most of Conway's conclusions regarding the wall-film. However, the precision of volume delivered obtained by Conway was relatively poor and therefore tended to obscure many effects that have been treated here. Thus, his conclusions about tip-technique, drainage, meniscus adjustment and the effects of holding the pipette by hand, although no doubt true in his work, are otherwise erroneous. Further, he suggested that the precision of delivery was proportional to the wall-film and hence to (delivery time)$^{-\frac{1}{2}}$. The work described here shows that the precision cannot be predicted from considerations of the wall-film alone.

The most serious sources of variable error with a typical pipette are, in order of magnitude—

(1) the vibrations caused by handling (larger pipettes only);
(2) the effect of temperature on the wall-film;
(3) a varying inclination (long drainage times only); a varying tip-technique; the thermal effect of handling;

all of which can be minimised by taking suitable precautions. If this is not done, the variation in the volume delivered can easily be more than ten times that quoted in Part I, *i.e.*, in round figures, the precision of delivery will be $\pm 0{\cdot}01$ to $\pm 0{\cdot}02$ ml depending on the size of the pipette. Such a variation may well be acceptable in practice, although it must be stressed that the differences in the volumes delivered from day to day may be greater.

Useful increases in precision and accuracy can be conveniently obtained by keeping the pipette vertical at all times, by clamping the larger pipettes during delivery, by handling the pipettes with insulating material, by applying corrections for the effect of temperature on the wall-film and by using a short drainage time. Much can be done by intelligent selection of the pipette, attention being directed to its shape and finish, the quality of the glass and the position, regularity and thickness of the mark. It is important that the delivery time should be within the limits prescribed by the National Bureau of Standards[12] (in our opinion the lower limit prescribed by the British Standards Institute[13] is too small).*

Provided that these conditions are observed, a reliable technique is that described below.

Attach to the top of the pipette a suitable "pneumatic-control device" (devices that include a large volume of air are not recommended), and clamp the pipette in a vertical position. Fill by suction to about 2 cm above the mark, and wipe around the tip with tissue. Adjust the meniscus to the mark by allowing liquid to flow out with the tip held against a clean wet surface at a large included angle. During this operation, the meniscus must be viewed against a suitable background. Sufficient attention is seldom directed to this point, whereas due care is usually taken when viewing the meniscus in a burette. We use a small white card with a black lower portion, attached to the pipette at the mark; any of the standard methods of reading a meniscus in a burette will probably serve equally well, provided that they are consistently used.

Allow the pipette to deliver freely, *i.e.*, with the tip in air, in a vertical position. When flow has ceased, allow 3 seconds for drainage and then *either* touch the tip against the surface of the liquid *or* expel the tip-volume by operating the "pneumatic-control device" with the tip held against the wall of the vessel. The second method must be used if delivery has been made into a vessel that contains another liquid.

Make corrections for the appropriate expansion effects of temperature and the effect of temperature on the wall-film. Corrections for the effect of atmospheric pressure are required only in calibration.

If the above procedure is adopted, then the precision of delivery quoted in Part I can be realised. The accuracy will also be high, *i.e.*, the mean corrected volume delivered will

* Since this paper was prepared, B.S. 1583 has been revised (1961). The short (3 second) drainage time has been adopted and the minimum delivery time for some pipettes has been increased.

show little variation with time. Thus, the 10-ml pipette that was used in this work was calibrated, after delivery as above, seven times at intervals over a period of 2 years; the average of the volumes delivered (corrected to 20° C) was 9·9965 ml and all the calibrations were within 0·002 ml of this value.

It will be noted that the recommended method includes allowing the pipette to deliver freely, whereas the British Standards Institute[13] stipulates delivery with the tip held against the wall of the receiving vessel. In our opinion, free delivery is better, because (*a*) the conditions at the tip are more reproducible and (*b*) it is easier to keep the pipette vertical with free delivery. The British Standards Institute also specifies a different method of treating the tip, *i.e.*, holding the tip against a wetted surface. This could possibly be as reproducible as our method, *if the included angle were rigidly standardised*, but no specific angle is stipulated.

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Iodimetric Methods of Assay for Penicillin in Fermented Medium

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Two iodimetric methods are described for determining penicillin in fermented broth. In the first method, most of the impurities are removed by solvent extraction and the effect of the remaining impurities on the absorption of iodine is rendered negligible by adjustment of pH; the standard error of a single determination is approximately 2 per cent. The second method requires use of penicillinase, but is more rapid than the first, as it is direct and does not involve solvent extraction. This method depends on preliminary iodination of the impurities, during which penicillin is unaffected; the standard error of a single determination is also approximately 2 per cent.

Various methods have been devised for assaying penicillin in fermented broth,[1 to 8] but we have found none of these to be sufficiently accurate or otherwise entirely satisfactory for the dual purposes of process control and assessment of experimental fermentations on a large

scale. During our work, we found that iodimetric assay[9,10,11] with inactivation by alkali could be applied to fermentation samples if two conditions were fulfilled, *viz.*—

(*i*) Most of the organic impurities were removed by solvent extraction.
(*ii*) The uptake of iodine by the remaining impurities, which otherwise interfered with the blank determination, was decreased to a minimum by adjustment of pH.

Experimental

One difficulty in developing a method for assaying fermented medium is that the penicillin contents of natural samples are unknown, and it is impracticable to prepare a fermented medium having a known content of penicillin. The use of an unfermented corn-steep liquor - sugar medium, with the addition of known amounts of penicillin, is not entirely satisfactory, as such a medium differs in composition from fermented samples. The closest approach to naturally fermented samples of known penicillin content appeared to be by the use of a partly fermented medium in which the penicillin had been destroyed. This was accomplished by slightly acidifying and then setting side overnight or heating at 120° C; after neutralisation, the complete destruction of the penicillin was demonstrated by microbiological plate assay. Tests by the proposed methods A and B gave apparent-penicillin contents of up to 1 and 0·5 per cent., respectively. These contents, however, were too small to be of significance and were ignored in subsequent work on the samples.

Known amounts of twice recrystallised sodium benzylpenicillin or potassium phenoxymethylpenicillin, indistinguishable in potency from the current British National Standards for penicillin by both iodimetric and microbiological assay, were added. These standard preparations have potencies of 1670 units per mg for sodium benzylpenicillin and 1695 units per mg for phenoxymethylpenicillin (1530 units per mg for the potassium salt).[12] Although the term "unit" relates strictly to biological activity, it has been widely used as a convenient expression of penicillin content in relatively low concentrations, particularly in fermented medium, and we have therefore used the term in this paper in the sense that 1 mg of sodium benzylpenicillin is equal to 1670 units and 1 mg of potassium phenoxymethylpenicillin is equal to 1530 units. These two values are almost exactly equivalent to each other when allowance is made for the difference between the molecular weights of the two compounds.

We found that the second condition mentioned above could be satisfactorily met by decreasing the pH of the blank to 3·6, the lowest value to which benzylpenicillin can be subjected for the time necessary to complete the blank determination without causing significant inactivation of the penicillin present. Thus, when a typical inactivated fermentation medium derived from corn-steep liquor and reducing sugars, devoid of penicillin, was extracted with butyl acetate at pH 2, most of the impurities remained in the aqueous phase; extraction of the butyl acetate solution with phosphate buffer solution yielded a relatively pure aqueous extract. Suitable aliquots of this extract were made alkaline with sodium hydroxide, acidified with hydrochloric acid and iodinated; the blank solutions were iodinated directly after adjustment of pH to different values between 3·0 and 6·4. The amount of 0·01 N iodine absorbed by the test solution was 1·9 ml, and the results for the blanks were—

pH	6·4	5·0	4·0	3·6	3·0
0·01 N iodine absorbed, ml	3·0	2·1	2·1	1·9	1·9

At pH 3·6 and below, the amounts of iodine absorbed by test and blank solutions were identical, so that there is no apparent interference from impurities at these values.

A pH of approximately 3·6 can be readily attained in practice under the proposed conditions of assay by adding 1 ml of 1·0 N acetic acid to 10 ml of 0·01 M phosphate buffer solution. The extent of inactivation of benzylpenicillin under these conditions was determined by measuring the absorption of iodine from a phosphate buffer solution containing 20,000 units of benzylpenicillin (much more than is normally present in a test) after a suitable addition of 0·02 N iodine had been made and the solution had been set aside at room temperature (approximately 21° C) for different periods. The excess of iodine was determined by titration with sodium thiosulphate solution; the results were—

Time of standing, minutes	0	5	10	15	20
Titre of 0·01 N sodium thiosuphate, ml	48·15	48·10	47·95	47·9	47·9

As the time needed for a blank determination is less than 5 minutes, the error arising from inactivation of the penicillin in the blank is negligible.

The extraction of penicillin from fermented medium at pH 2 into solvent is generally carried out under ice-cold conditions, because of the instability of benzylpenicillin in acid solution. However, such cooling of solutions considerably lengthens the assay and is therefore undesirable in a method intended for routine tests on large numbers of samples; extraction at room temperature was therefore considered. This appeared to be practicable, as transfer of penicillin to the solvent can be carried out so rapidly that the likelihood of significant inactivation during extraction seemed small, and, once in butyl acetate solution, the penicillin is sufficiently stable to withstand any normal delays in the subsequent assay; tests for stability indicated a maximum loss of 0·2 per cent. per hour. Experiments on the efficiency of the solvent extraction at room temperature were therefore carried out on inactivated fermented medium containing known added amounts of sodium benzylpenicillin. The mean potency of the samples was found from twenty-two determinations to be 1160 units per ml, compared with a theoretical value of 1166. This is equivalent to 99·5 per cent. of the original activity (or at least to 98·5 per cent., allowing for the possible small blank of the medium used), *i.e.*, solvent extraction can be satisfactorily carried out at room temperature.

The iodine equivalents of sodium benzylpenicillin and potassium phenoxymethylpenicillin were determined in solution in 0·01 M phosphate buffer under the assay conditions used; 1 ml of 0·01 N iodine was found to be equivalent to 0·395 mg (660 units) of sodium benzylpenicillin and to 0·418 mg (640 units) of potassium phenoxymethylpenicillin. Under these conditions, the iodine equivalents were not found to vary significantly with concentration, provided that not more than half of the iodine present was absorbed.[13,14]

The method finally developed is described below. We have included it in this paper because it is a highly satisfactory assay procedure that can be used if penicillinase is unavailable and also because it may be useful when information is required about penicillinase-resistant and penicillinase-sensitive penicillins.

Method A: determination of benzyl- or phenoxymethylpenicillin in fermented medium after extraction and alkaline hydrolysis

Reagents—

Iodine, 0·02 N—Freshly prepare this solution each day from 0·1 N iodine containing 2 per cent. of potassium iodide.

Phosphate buffer solution, 0·02 M (*pH* 7·0)—Prepare from the sodium salts.

Sodium thiosulphate, 0·01 N—Accurately standardised.

Procedure—

Filter the sample, accurately transfer (by pipette) 10 ml of the filtrate, or of a suitable dilution if the potency exceeds 4000 units per ml, to a separating funnel, and add approximately 50 ml of butyl acetate. Add 1·0 ml of 8 per cent. v/v sulphuric acid containing 4 per cent. of cetrimide, shake vigorously for 30 seconds, and allow to separate. Discard the lower aqueous layer, and wash the butyl acetate layer with 10 ml of water. Add 75 ml of the phosphate buffer solution, shake vigorously for 30 seconds, allow to separate, and run the lower aqueous layer into a 250-ml calibrated flask. Rinse the separating funnel with 50 ml of the buffer solution, add the rinsings to the contents of the flask, and dilute to the mark with water. Transfer a 20-ml portion of this solution to a 150-ml stoppered flask, add 5 ml of 1·0 N sodium hydroxide, and set aside for 5 minutes. Acidify with 5 ml of 1·1 N hydrochloric acid, add 5 ml of 0·02 N iodine, and set aside for 5 minutes (exclude light during iodination). Titrate the excess of iodine with 0·01 N sodium thiosulphate added from a 10-ml burette graduated in 0·01-ml divisions; use 1 ml of a 1 per cent. solution of starch as indicator.

To determine the blank value, transfer a 20-ml portion of the buffer solution to a flask, add 2 ml of 1·0 N acetic acid, and mix. Add 5 ml of 0·02 N iodine, and immediately titrate with 0·01 N sodium thiosulphate; add 1 ml of the starch solution near the end-point. If difficulty is experienced in ascertaining the end-point, carbon tetrachloride should be used as indicator.

Calculate the potency of the sample from the equation—

$$\text{Potency, units per ml} = \frac{(B - A) \times F \times E \times 250}{\text{Volume of sample, ml} \times 200}$$

in which A and B are the volumes of 0·01 N sodium thiosulphate needed for the test and

blank solutions, respectively, F is the factor of the sodium thiosulphate solution and E is 660 for benzylpenicillin or 640 for phenoxymethylpenicillin.

ACCURACY OF METHOD—

Determinations on samples prepared for experiments on the efficiency of extraction indicate that the method gives results close to the theoretical values; in fact, results are low by about 0·5 to 1·5 per cent. The reproducibility of the method is good, the standard error of a single determination being approximately 2 per cent. This is based on the results in Table I for replicate tests on several samples of fermented media.

TABLE I

POTENCIES FOUND FOR FERMENTED SAMPLES IN REPLICATE DETERMINATIONS BY METHOD A

Tests Nos. 1 and 2 were carried out by one operator and tests Nos. 3 and 4 by a second operator

Sample No.	Potency found in— test No. 1, units per ml	test No. 2, units per ml	test No. 3, units per ml	test No. 4, units per ml
1	1540	1560	—	—
2	1440	1370	—	—
3	1830	1790	—	—
4	1680	1700	—	—
5	1890	1900	—	—
6	1720	1760	—	—
7	1350	1330	—	—
8	1690	1730	—	—
9	1560	1570	—	—
10	780	790	—	—
11	1990	2000	—	—
12	1600	1660	—	—
13	1630	1590	—	—
14	1270	1280	1320	1300
15	1270	1270	1260	1250
16	1570	1550	1610	1600
17	1640	1660	1550	1510
18	1780	1780	1820	1790

As a further check on accuracy, samples of fermented medium were assayed by both method A and a cylinder - plate method with *Bacillus subtilis*; for the latter procedure six plate assays incorporating eighteen cylinders of sample and eighteen of penicillin standards (2, 4 and 8 units per ml) were used. The standard error of a single test was approximately 4 per cent. Tests on twenty-four samples gave a mean potency of 1827 units per ml by the *B. subtilis* method and 1841 units per ml by the iodimetric assay. The closeness of these figures can be regarded as satisfactory evidence of the accuracy of method A.

DEVELOPMENT OF DIRECT ASSAY

Further work on simplification of the iodimetric method, with the particular object of avoiding the extraction procedure, proved abortive, owing to interference from the relatively large amount of soluble organic matter present in the samples, which led to excessively high blanks. A new approach to the problem was therefore made, in which elimination of interference from iodine-absorbing impurities was attempted by first treating the sample with iodine solution, thereby iodinating the impurities, but leaving the penicillin unchanged; the penicillin was subsequently determined by a hydrolytic iodination procedure. At the same time, efforts were made to devise a method of adequate accuracy, incorporating all the most desirable features needed in a method to be used for plant control and for assaying experimental laboratory fermentations and flask cultures. These features, which were successfully incorporated into the method, were—

(*i*) Use of a minimum amount of sample, so as to permit (*a*) small-scale work to be evaluated and (*b*) a filtered portion of sample to be rapidly obtained.
(*ii*) Avoidance of a solvent-extraction procedure, as this is time-consuming.
(*iii*) Simplicity of procedure and instruments.
(*iv*) Specificity.[15,16]

(*v*) Stability of reagents.
(*vi*) Applicability, without modification, to fermentation samples having very varied compositions.

The more important factors involved in the method are discussed below.

EFFECTS OF CONSTITUENTS OF FERMENTATION MEDIUM ON DIRECT ASSAY—

The effects of impurities on iodimetric assay involving inactivation by alkali have been studied by Wild[11]; he used crude samples of penicillin salts having low potencies and concluded that the method was not entirely satisfactory, owing to absorption of iodine by the impurities, the difficulty in accurately compensating for this absorption in the blank and the change in absorption of iodine by the impurities after alkaline hydrolysis, which again could not be compensated for in the blank. The same problem presents itself with fermented media, but in greater degree, as impurities are present in larger proportion than in crude penicillin salts. Tests confirmed the impracticability of applying an iodimetric assay involving inactivation by alkali directly to fermented media.

It was apparent, however, that the errors arising from inactivation might be eliminated or decreased by using penicillinase instead of alkali; because of its specific action, penicillinase should inactivate penicillin without having any effect on the other constituents. However, even though the absorption of iodine by these constituents and by the penicillinase can be determined by blank tests, the values are high in relation to the absorption of iodine by the penicillin after inactivation; further, apart from the undesirability of high blanks, we have been unable to obtain accurate results by this procedure.

COMBINATION OF TEST AND BLANKS—

In order to avoid difficulties from the impracticability of subjecting both test and blank to the same treatment, we tried a radical departure from the usual iodimetric procedure by combining the blank and test. This was possible because iodine was absorbed not by penicillin, but only by the products of its inactivation. For this purpose, the sample was treated with iodine solution at pH 7·0 to iodinate the impurities as far as was practicable. The absorption of iodine increases with increase in pH, but a pH of 7·0 cannot be exceeded without introducing error from inactivation of the penicillin. The excess of iodine was destroyed by titration with sodium thiosulphate solution, and penicillinase was then added to inactivate the penicillin. Acidification of the solution before iodination of the products of inactivation rendered further absorption of iodine by the impurities negligible. This is essential, as the amount of iodine absorbed at this stage provides the measure of the penicillin in the sample.

DEGREE OF ACIDIFICATION—

In preliminary experiments it was found for some samples, particularly those of low potency taken early in the fermentation, that, if the test solution was rendered too acid after the preliminary iodination, some iodine was released; this led to low results. The pH in these tests was about 1·3, which is produced when about 5·5 ml of 1 N hydrochloric acid are added. A pH value of about 2 is produced with most samples when 5 ml of 0·2 N hydrochloric acid are added to the test solution. Tests were therefore carried out to determine the effect of pH; thirty-four samples were analysed by the direct procedure at pH values of 1·3 and 2·0 and also by method A. The mean potencies found by the direct method were 3360 (at pH 1·3) and 3560 units per ml (at pH 2·0), and that found by method A was 3580 units per ml. These results show that there is good agreement between results by the extraction method and the direct method carried out at pH 2.

PENICILLINASE—

Penicillinase can be prepared by the general method described in the British Pharmacopoeia, but other procedures have been described.[17 to 21] However, for assay purposes it is essential to use a highly potent[16] preparation, and we have used nutrient-broth filtrates prepared from *Bacillus cereus*. These had potencies of at least 120,000 units per ml per hour at 20° C, and it was unnecessary to concentrate or purify them further. They would, of course, permit the growth of contaminating micro-organisms, which in turn would cause rapid destruction of the enzyme. It was therefore necessary to add some suitable preservative that would not lead to chemical destruction of the enzyme and would also not interfere

with the assay of penicillin. Both toluene and chloroform were found to be suitable for this purpose, and chloroform was more convenient to use, as excess of it sinks to the bottoms of the containers. There was no significant loss of potency after the solution had been stored at 5° C for 5 weeks.[21]

IODINE EQUIVALENT OF PENICILLIN—

Since the mode of inactivation of penicillin by penicillinase cannot be identified with alkali inactivation, it was necessary to determine the iodine equivalent of penicillin under the proposed standard test conditions. This was carried out for benzylpenicillin and phenoxymethylpenicillin as before, recrystallised sodium benzylpenicillin (potency 1670 units per mg) and potassium phenoxymethylpenicillin (potency 1530 units per mg) being used.

It was found that the iodine equivalent varied with the concentration and type of penicillin used in the test.[13] When the amounts of iodine absorbed were plotted against the concentration of penicillin used, the regression lines were only slightly cuved at high concentrations of penicillin, but considerably curved at lower concentrations, at which the test is not usually applied. The results obtained for the two penicillins are shown in Table II.

TABLE II

IODINE EQUIVALENTS OF PENICILLINS AT VARIOUS CONCENTRATIONS

Benzylpenicillin		Phenoxymethylpenicillin	
Units present per test	Units equivalent to 1 ml of 0·01 N iodine	Units present per test	Units equivalent to 1 ml of 0·01 N iodine
880	521	860	541
1330	556	1290	566
1770	572	1720	567
2210	577	2150	567
4420	595	4310	582
6630	606	6470	592
8840	612	8630	598
11,050	627	10,790	606
13,270	635	12,900	611

TIME REQUIRED FOR INACTIVATION BY PENICILLINASE—

Various batches of penicillinase were examined to ascertain that the preferred amount of penicillinase, 0·5 ml, would cause complete inactivation of the optimum amount of penicillin for the test in a suitable short time. The results are shown in Table III, from which it can be seen that inactivation occurs within 5 minutes under the conditions used; however, in order to have an adequate margin of safety we decided to allow 10 minutes for inactivation.

TABLE III

INACTIVATION OF PENICILLINS BY PENICILLINASE

Penicillinase used				
Batch No.	Amount present in 0·5 ml, units	Period allowed for inactivation, minutes	Penicillin added, units	Penicillin found, units
Benzylpenicillin—				
44/5	130,000	10	12,400	11,700
		20		11,800
		30		11,900
		40		11,800
D6	60,000	5	9900	10,200
		10		10,000
		20		9900
44/5	130,000	10	9900	10,000
		15		
Phenoxymethylpenicillin—				
D7	80,000	5	11,400	11,700
		10		11,400
		15		11,500
42/6	245,000	5	6600	6700
		20		
D6	60,000	5	11,300	11,300
		10		

INDICATOR—

In the extraction procedure, starch is normally used as indicator, but we found for some experimental fermentations that the end-point was unsatisfactory. In these instances, however, carbon tetrachloride could be used satisfactorily, and we therefore adopted its use in the direct method so as to make the procedure applicable to all samples.

FIRST IODINATION PERIOD—

In order to fix a suitable period for the initial iodination of the broth at pH 7·0, tests were made with iodination periods of 5 and 15 minutes; the results are shown in Table IV. As there was no significant difference between the results after iodination for 5 and 15 minutes, the shorter time was adopted for the test.

TABLE IV

POTENCIES FOUND AFTER DIFFERENT PERIODS OF INITIAL IODINATION

Sample No.	Potency found by direct method after iodination at pH 7 for— 5 minutes, units per ml	15 minutes, units per ml	Potency found by extraction method, units per ml
V67	2900	2920	2960
V44	1440	1420	1300
V43	2120	2140	2270
V44	1990	2060	2070
V58	1950	1950	1970

TABLE V

RESULTS FOUND FOR PREPARED SAMPLES BY DIRECT METHOD

The volume of each sample used was 2 ml

Type of medium	Theoretical penicillin content of sample, units	Penicillin found, units	Difference from theoretical content, %
Benzylpenicillin—			
Acid-inactivated	8730	8600	−1·5
		8700	−0·3
Unfermented	8730	8400	−3·8
Unfermented	11,040	11,300	+2·4
		11,200	+1·4
		11,200	+1·4
		11,000	−0·4
		11,100	+0·5
		11,000	−0·4
Phenoxymethylpenicillin—			
Acid-inactivated	10,800	10,800	0·0
		10,700	−0·9
		10,700	−0·9
		10,900	+0·9
		10,600	−1·9
		10,700	−0·9
		10,700	−0·9
		10,700	−0·9
		10,600	−1·9
		10,900	+0·9
		10,900	+0·9
		11,000	+1·9
		10,900	+0·9
		11,000	+1·9
		11,000	+1·9
		11,000	+1·9
		10,900	+0·9
		10,900	+0·9
Acid-inactivated	8450	8200	−3·0
Unfermented	10,810	10,900	+0·8
		10,800	−0·1
		10,800	−0·1
		10,800	−0·1
		10,900	+0·8
		10,800	−0·1

ASSAY OF SAMPLES HAVING KNOWN PENICILLIN CONTENTS—

Checks of the final direct procedure were made on samples of unfermented or inactivated media containing known added amounts of penicillin prepared as before. The results of these tests are shown in Table V and are close to the theoretical values. The mean result for benzylpenicillin is low by 0·08 per cent.; that for phenoxymethylpenicillin is high by 0·12 per cent. These differences are sufficiently insignificant as to need no correction in normal experimental or plant-control work.

A further check on accuracy was made by assaying sixteen samples of fermented medium by the direct iodimetric procedure and by a cylinder-plate assay with *B. subtilis*. The mean of the results by the iodimetric procedure was 1·9 per cent. below that of those by the *B. subtilis* method.

APPLICABILITY—

Samples having high potency—The direct method is satisfactory for samples having potencies up to 6000 units per ml. For samples of higher potency, it is necessary to use a proportionately smaller amount of sample than 2 ml or to use 2 ml of a suitable dilution; otherwise, the excess of iodine present is insufficient for complete iodination.

Samples having low potency—Assays of samples taken early in the course of fermentation are not usually required. However, if such assays are needed, care is necessary if high concentrations of certain unfermented constituents of the medium, *e.g.*, corn-steep liquor and sugars, are present, as a small amount of absorbed iodine is sometimes released at the acidification stage of the assay. If this occurs, a blank test must be carried out to measure the amount of iodine released, a correction for which must be applied in the normal test.

REPRODUCIBILITY—

Replicate tests were made by different operators on fifty-two samples of fermented medium over a short period; the standard error of the results was 1·9 per cent.

SPEED—

A skilled operator can carry out a single test in about 30 minutes from the receipt of the sample; this represents the minimum time in which a single result can be made available. However, since much of the period is "waiting time," about six samples of filtered broth per hour can be tested simultaneously (this does not allow for the preparation of reagents and reporting of results). The method is also economical in terms of apparatus and reagents.

METHOD B: DIRECT DETERMINATION OF BENZYL- OR PHENOXYMETHYLPENICILLIN IN FERMENTED MEDIUM WITH USE OF PENICILLINASE

REAGENTS—

Penicillinase—This must have a potency of not less than 120,000 units per ml per hour at 20° C.

Iodine, 0·02 N—As for method A.

Sodium thiosulphate, 0·01 N—As for method A.

Phosphate buffer solution, 0·01 M (*pH* 7·0)—Prepare from the sodium salts.

PROCEDURE—

Accurately transfer, by pipette, 2 ml of filtered sample to a 150- or 250-ml conical flask fitted with a glass stopper. Add about 20 ml of 0·01 M phosphate buffer solution and approximately 5 ml of 0·02 N iodine, and set aside for 5 minutes. Destroy the excess of iodine by titration with 0·01 N sodium thiosulphate; use 10 ml of distilled carbon tetrachloride as indicator, and take care not to add excess of the thiosulphate solution. Add 0·5 ml of penicillinase, mix gently, and set aside at room temperature for 10 minutes. Acidify with 5 ml of 0·2 N hydrochloric acid, mix gently, and accurately add 20 ml of 0·02 N iodine. Mix thoroughly, but do not shake (*i.e.*, avoid excessive transfer of iodine to the carbon tetrachloride), set aside in the dark for 5 minutes, and then immediately titrate with 0·01 N sodium thiosulphate.

To determine the blank value (the amount of iodine absorbed by the penicillinase used in the test), proceed exactly as described above, but omit the 2-ml portion of sample; this need be done only once for each batch of penicillinase.

CALCULATION OF RESULTS—

The potency of the fermented broth is calculated from the equation—

$$\text{Potency, units per ml} = \frac{(S - A - P) \times F \times E}{2}$$

in which A is the volume of 0·01 N sodium thiosulphate required for the test solution, F is the factor of the sodium thiosulphate solution, S is the volume of 0·01 N sodium thiosulphate equivalent to 20 ml of the 0·02 N iodine, P is the difference (in millilitres) between the volume of 0·01 N sodium thiosulphate required to neutralise 20 ml of 0·02 N iodine and that required in the blank determination and E is the penicillin equivalent (in units) of 1 ml of 0·01 N iodine.

However, as shown in Table II, the value of E varies with the type of penicillin assayed and also with the concentration of penicillin in the sample, so that simple application of the above equation is impracticable. For general ease of calculation, it is convenient to construct Tables so that the value of $\frac{(S - A - P) \times E}{2}$ can be readily determined. Since variations in penicillin equivalent often occur between laboratories, individuals may prefer to make their own assessments, but, if the results in Table II are used, the penicillin content of a sample is related to iodine absorbed as shown in Table VI. (These values have been used for calculating the results in this paper.)

TABLE VI

RELATIONSHIP BETWEEN IODINE ABSORBED AND CONCENTRATION OF PENICILLIN IN SAMPLE

Volume of 0·01 N iodine absorbed ml	Concentration of penicillin in sample	
	Benzylpenicillin, units per ml	Phenoxymethylpenicillin, units per ml
1	270	270
2	550	550
3	840	840
4	1150	1140
5	1450	1440
6	1770	1730
7	2080	2040
8	2400	2340
9	2710	2640
10	3030	2940
11	3340	3250
12	3660	3560
13	4000	3870
14	4330	4190
15	4660	4500
16	4990	4830
17	5320	5150
18	5650	5460
19	5980	5780

NOTES ON METHOD B—

Samples taken in early stages of fermentation—Large amounts of unfermented matter are present in samples taken in the early stages of fermentation, and a small amount of iodine is sometimes liberated at the acidification stage of the assay; this is shown by the development of a pink colour in the carbon tetrachloride. With such samples, set the solution aside for 5 minutes after acidification, complete the test in the usual way, and carry out a blank determination as described below.

By pipette, place 2 ml of the sample in a 150- or 250-ml flask fitted with a glass stopper, and add about 20 ml of 0·01 M phosphate buffer solution. Add 0·5 ml of penicillinase, set aside for 10 minutes at room temperature, add excess of 0·02 N iodine (approximately 10 ml), and set aside for 5 minutes. Destroy the excess of iodine by titration with 0·01 N sodium thiosulphate; use 10 ml of carbon tetrachloride as indicator, and take care not to add excess of the sodium thiosulphate solution. Acidify with 5 ml of 0·2 N hydrochloric acid, shake gently, set aside for 5 minutes, and titrate with 0·01 N sodium thiosulphate.

Let the blank titre of 0·01 N sodium thiosulphate be B ml. Add this result to the test titre, so that the final absorption titre becomes (S — A — P + B) ml, and calculate the potency as above.

Treatment of apparatus—All apparatus that has contained penicillinase must be steam-heated in an autoclave or heated at 150° C after use to destroy any residual enzyme.

Conclusions

Benzyl- or phenoxymethylpenicillin can be determined iodimetrically in fermented medium if (*a*) partial purification is first carried out by extracting the penicillin into butyl acetate and then into phosphate buffer solution and (*b*) the blank in the iodination procedure is adjusted to pH 3·6. At this pH inactivation of the penicillin is negligible, and the uptake of iodine by the residual impurities is small and equal to the uptake in the test.

These penicillins can also be determined directly and more rapidly, without extraction, by preliminary iodination of the sample; the impurities, but not the penicillin, are iodinated.

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The Trace Determination of Phenyl- and Methylmercury Salts in Biological Material

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A method is described for determining phenyl- and methylmercuric ion in organic material. The sample is made strongly acid with hydrochloric acid and extracted with benzene, and the organic mercury is then re-extracted with aqueous sodium sulphide. After oxidation with acid permanganate, mercury is determined in this solution by a titrimetric procedure; inorganic mercury does not interfere. Recoveries of about 90 per cent. have been obtained when 2 and 20 p.p.m. of phenyl- and methylmercury acetates were added to biological material. The method is capable of measuring concentrations of mercurials well below 1 p.p.m.

An investigation in this laboratory into the metabolic fate of phenyl- and methylmercury salts required a method of determining trace amounts of these substances in animal tissue in the presence of inorganic mercury. Methods are available for examining crops treated

with organic mercurial fungicides, but these are restricted to the determination of total mercury. More specific methods have been suggested, based on ultra-violet spectrophotometry[1] and polarography,[2] but these have not been elaborated for a range of biological materials. Miller, Lillis and Csonka[3] have described a method in which the sample is oxidised with alkaline permanganate, after which an arylmercury salt may be extracted with a solution of dithizone in chloroform. This method is not very sensitive, as the yellow colour of the complex formed from dithizone and phenylmercuric ion is not intense, and it is necessary to measure the green colour of the unchanged dithizone. Moreover, the method is stated to be unsuitable for alkylmercury salts.

A method for determining phenyl- and methylmercury salts in biological material has been developed in which the substance is extracted by benzene from a homogenate of the sample that has been made strongly acid with hydrochloric acid. The benzene solution is shaken with a dilute aqueous solution of sodium sulphide, with which these organic mercurials react to form water-soluble complexes.[4] The aqueous layer is then oxidised with acid permanganate, and the mercury content is determined by a modification of Fabre, Truhaut and Boudène's highly sensitive titrimetric method.[5]

Method

Reagents—

Sodium sulphide solution, 1 *per cent. w/v.*

Hydroxylamine hydrochloride solution, 20 *per cent. w/v.*

Urea solution, 10 *per cent. w/v.*

EDTA solution—Prepare a 2·5 per cent. w/v solution of the disodium salt of ethylenediaminetetra-acetic acid.

Sodium acetate solution, 50 *per cent. w/w*—Prepare from sodium acetate trihydrate.

Dithizone solution—Prepare a stock solution containing 20 mg of dithizone in 100 ml of carbon tetrachloride; store this solution in a refrigerator. Prepare the working solution when required by diluting 1 ml of the stock solution to 20 ml with chloroform.

Standard mercury solution—Prepare a stock solution by dissolving 67·7 mg of mercuric chloride in 1 litre of 0·5 N hydrochloric acid. For use, dilute 1 ml of the stock solution to 50 ml with water; this solution contains 1 μg of mercury per ml.

Extraction of organic mercury—

Prepare a homogenate containing 5 per cent. of the tissue in water, measure 20 ml into a stoppered tube, and add 4 ml of hydrochloric acid, sp.gr. 1·18, and 20 ml of benzene. Shake the tube for 5 minutes, allow the layers to separate, and spin in a centrifuge if necessary to obtain a clear upper layer. Transfer 15 ml of the upper benzene layer to a second stoppered tube, add 10 ml of sodium sulphide solution, and shake for 5 minutes. Allow the layers to separate, spin in a centrifuge if necessary to obtain a clear lower layer, and transfer the lower layer as completely as possible to a third stoppered tube. Shake the benzene layer with 5 ml of water, allow to separate, and add the lower aqueous layer to the contents of the third tube.

Oxidation with acid permanganate—

To the aqueous sulphide solution add 2 ml of 50 per cent. v/v sulphuric acid, 0·5 g of potassium permanganate and 1 drop of octanol. Place the loosely stoppered tube in a boiling-water bath for 1 hour. Allow to cool a little, add 3 ml of hydroxylamine hydrochloride solution to decolorise, and cool to room temperature.

Determination of mercury—

To the oxidised solution add 2 ml of urea solution and 1 ml of EDTA solution, adjust the pH to 1·5 with sodium acetate solution (use an external indicator paper), and add 0·5 ml of chloroform; this is the test solution. Prepare a control solution by mixing 15 ml of water, 2 ml of 50 per cent. v/v sulphuric acid, 0·5 g of potassium permanganate, 3 ml of hydroxylamine hydrochloride solution and the remainder of the reagents as above; adjust to the same pH.

From the burette, add the diluted dithizone solution in small portions to the test solution; replace the stopper, and shake the tube vigorously after each addition. If mercury is present, the separating organic layer will have an orange colour. Continue the titration until the chloroform layer has a greyish colour, intermediate between the orange of the mercury complex

and the green of unchanged dithizone. Record the volume of dithizone solution used, and add this same volume to the control solution. Titrate the control solution with standard mercury solution; add the titrant in small portions, with vigorous shaking after each addition, until the colour of the chloroform layer that separates matches that of the layer in the test solution. The volume (in millilitres) of standard mercury solution used is equal to three-quarters of the amount of organic mercury (in micrograms) present in the original sample.

Results and discussion of the method

To determine the recoveries of phenyl- and methylmercury salts by the proposed method, known amounts of phenyl- and methylmercury acetates were added to homogenates of normal rat tissue or to rat urine. Solutions containing 1 mg of the acetates per ml were prepared in acetone, and these were diluted with water just before addition to the samples. The results in Table I show that recoveries were low by up to 15 per cent. and that this deficit does not seem to be affected by the concentration of the mercury compound in the tissue; the recovery from liver was the same at concentrations of 2 and 20 p.p.m. The method is sufficiently accurate for metabolic studies on organic mercurials and is capable of measuring concentrations well below 1 p.p.m.

Table I

Recovery of phenyl- and methylmercury acetates added to rat tissue and urine

		Methylmercury acetate			Phenylmercury acetate		
Sample	Amount of sample taken	Amount added, μg	Amount found, μg	Recovery, %	Amount added, μg	Amount found, μg	Recovery, %
Liver	0·5 g	10	9·2	92	10	8·8	88
Liver	1·0 g	2	1·8, 1·9	91	2	1·75, 1·75	88
Brain	1·0 g	2	1·8, 1·7	87	2	1·75, 1·65	85
Kidney	1·0 g	2	1·8, 1·8	90	2	1·85, 1·75	90
Urine	10 ml	10	8·8	88	10	9·5	95

Inorganic mercury present in the tissues is not extracted into benzene under the conditions of the method and does not interfere. It has not usually been found practicable to measure the amount of inorganic mercury in the sample by analysis of the aqueous layer from the first extraction with benzene, as this contains a proportion of the organic mercury dissolved in the benzene, which remains emulsified and is not easy to separate. If the ratio of inorganic to organic mercury in the sample is large, the error from the residual organic mercury in this layer may not be significant, or it may be possible to remove the interference by washing the aqueous layer with benzene. In general, however, it is more convenient to determine the total-mercury content of a second sample and to obtain the inorganic-mercury content by difference.

Although the proposed method has been developed for determining phenyl- and methylmercury salts in animal tissues, there seems to be no reason why it should not be applicable to the determination of alkyl- and arylmercury compounds in vegetable samples.

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The Spectrophotometric Determination of Sulphadiazine, Sulphamerazine and Sulphathiazole in Mixtures

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A rapid method is described for the spectrophotometric determination, without prior separation, of sulphadiazine, sulphamerazine and sulphathiazole present in one solution. Sulphadiazine is determined by the Association of Official Agricultural Chemists' method, which involves use of 2-thiobarbituric acid and measurement at 532 mμ of the colour developed. Direct optical-density measurements on a solution of the mixture in hydrochloric acid are made at 243 and 280 mμ, and the sulphamerazine and sulphathiazole contents are then calculated by solving simultaneous equations in which the measured values of $E^{1\%}_{1\,cm}$ and the sulphadiazine content found have been substituted. The method has been applied to a range of prepared and commercial mixtures.

THE use of sulphonamide mixtures has been fairly common since sulphonamide therapy was first introduced, and the problem of determining individual components in such mixtures has been tackled in several ways. One frequently used approach consists in paper-chromatographic separation and subsequent titrimetric or colorimetric determination of the separated sulphonamides. Two official methods[1,2] are based on this type of procedure; the technique is naturally somewhat lengthy, and results are not accurate unless averages of multiple determinations are taken.

One common mixture of sulphonamides contains sulphadiazine, sulphamerazine and sulphathiazole, and numerous preparations incorporating these three compounds are available in various countries. The British Pharmaceutical Codex[3] lists a formula for trisulphonamide tablets consisting of these three components, but the assay method described is for total-sulphonamide content only. The object of this work was to find a rapid and reasonably accurate method for determining the individual components of preparations containing these three sulphonamides without prior separation.

In the past, single sulphonamides have been determined by making use of their pronounced absorption maxima in the ultra-violet region.[4] Banes[5] took advantage of the particularly favourable difference between the absorption spectra of sulphadiazine and sulphathiazole in acid; he devised a simultaneous spectrophotometric method based on measuring the optical densities of the solutions at 239 and 280 mμ, at which the two components have their absorption maxima, and solving simultaneous equations in the usual manner. Application of a similar procedure to a system also containing sulphamerazine was prevented by the fact that sulphadiazine and sulphamerazine have closely similar spectra.

To overcome this difficulty, Thomas and Lagrange[6] proposed a method based on the colours produced when the aminopyridine groups of sulphamerazine and sulphadiazine were allowed to react with resorcinol and sulphuric acid; these colours had absorption maxima at different wavelengths. Sulphathiazole was determined by direct optical-density measurements at 280 mμ in hydrochloric acid, and corrections were made for sulphadiazine and sulphamerazine. However, when this method was tried, Thomas and Lagrange's claims as to stability of solutions and lack of interference by sulphathiazole could not be substantiated, and the precision and accuracy were poor; further, two different solutions of the sample were needed, which fact did not make the method rapid.

Attention was then turned to the Association of Official Agricultural Chemists' method[7] for determining sulphadiazine in the presence of other sulphonamides by means of its colour reaction with 2-thiobarbituric acid. In mixtures of sulphadiazine and sulphamerazine, the latter is determined by measuring the total absorption in 0·1 N hydrochloric acid and then subtracting the absorption due to sulphadiazine (known from its colorimetric determination). It seemed reasonable to suppose that, by a combination of this method with that described by Banes,[5] the three sulphonamides could be determined in the same solution.

EXPERIMENTAL

All optical-density measurements were made in matched 1-cm fused-silica cells with a Beckman DU spectrophotometer, and a cell correction was applied when necessary. The

ultra-violet spectra were plotted for solutions of the sulphonamides in 1 and 0·1 N hydrochloric acid, and little difference was found between the results at these two concentrations of acid. For convenience of combination with the thiobarbituric acid method for sulphadiazine, 0·1 N hydrochloric acid was used. The absorption spectra for the three sulphonamides at a concentration of 10 μg per ml (1 mg per 100 ml) are shown in Fig. 1. Maximum absorption was at 243 mμ for sulphadiazine and sulphamerazine and at about 280 mμ for sulphathiazole. With the instrument used, the values found for $E^{1\%}_{1\,cm}$ were—

	Sulphadiazine	Sulphamerazine	Sulphathiazole
$E^{1\%}_{1\,cm}$ at 243 mμ	568	579	119
$E^{1\%}_{1\,cm}$ at 280 mμ	119	128	492

Additivity of optical densities was ascertained, and solutions of all three sulphonamides were found to obey the Beer - Lambert law at the wavelengths indicated above; for concentrations of sulphonamide between 0 and 25 μg per ml (0 to 2·5 mg per 100 ml), graphs of concentration against optical density were linear and passed through the origin.

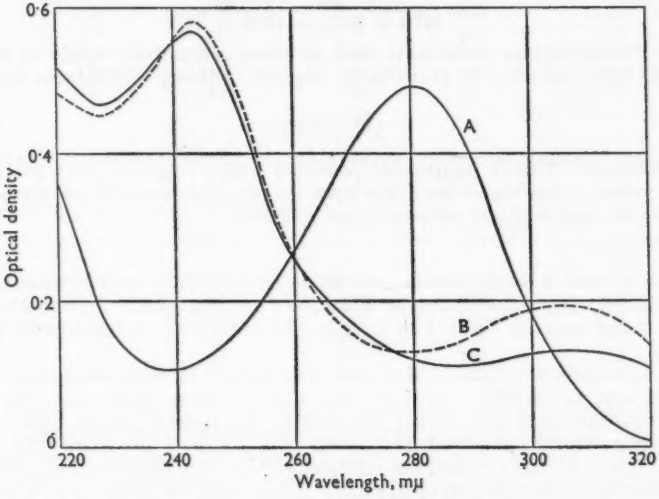

Fig. 1. Ultra-violet absorption spectra of solutions containing 1 mg of sulphonamide per 100 ml in 0·1 N hydrochloric acid: curve A, sulphathiazole; curve B, sulphamerazine; curve C, sulphadiazine

Tests of the A.O.A.C. thiobarbituric acid method for sulphadiazine confirmed all the findings of a previous collaborative study.[8] With the reagents and apparatus used, maximum absorption of the colour developed was at 533 instead of 532 mμ, and recrystallisation of the 2-thiobarbituric acid was unnecessary. Instead of glass-stoppered tubes, which have to be weighed before and after heating, it was found more convenient to use 10-ml calibrated flasks. A 1-ml portion of the sample or standard solution was placed in the flask, the contents of which were then diluted to the mark with buffered thiobarbituric acid solution. The flask was placed in a bath of boiling water, and, when the solution had attained the temperature of the bath, the stopper was inserted; the solution was then heated in the bath for 1 hour. When the flask was cool, any moisture lost was replaced by diluting to the mark with water. The amount of reagent present in relation to the amount of sample solution was therefore slightly less than in the A.O.A.C. method, but this did not affect reliability in any way. The influence of extraneous factors on development of colour and extinction coefficient was overcome by including standards with every batch of sample solutions, and results were calculated as in the A.O.A.C. method.[7] No interference from sulphamerazine or sulphathiazole was noticed when these compounds were present in proportions likely to be encountered in commercial mixtures.

If the optical densities of a solution containing sulphadiazine, sulphamerazine and sulphathiazole in 0·1 N hydrochloric acid at 243 and 280 mμ are S_{243} and S_{280}, respectively, then, since the optical density of the mixture is equal to the sum of those of its constituents—

$$1000S_{243} = 568d + 579m + 119t$$
$$1000S_{280} = 119d + 128m + 492t$$

where d, m and t are the concentrations of sulphadiazine, sulphamerazine and sulphathiazole, respectively, in milligrams per 100 ml. The value of d is known from the determination of sulphadiazine, so that, if $(1000S_{243} - 568d)$ and $(1000S_{280} - 119d)$ are denoted by S'_{243} and S'_{280}, respectively, the above equations can be written as—

$$S'_{243} = 579m + 119t$$
$$S'_{280} = 128m + 492t$$

from which the values of m and t can be calculated.

$$m = \frac{119S'_{280} - 492S'_{243}}{(128 \times 119) - (579 \times 492)}$$

$$t = \frac{579S'_{280} - 128S'_{243}}{(579 \times 492) - (128 \times 119)}$$

The values of the extinction coefficients used in these calculations apply to one particular instrument and time and must be periodically checked, although differences should be small.

METHOD

APPARATUS—

Spectrophotometer—Use an instrument provided with a tungsten lamp for measurements in the visible region, a hydrogen-discharge tube for measurements in the ultra-violet region and fused-silica or quartz 1-cm cells matched in pairs.

REAGENTS—

Citrate buffer and 2-thiobarbituric acid solutions—Prepare as described[7] in A.O.A.C. method No. 32.285; recrystallisation of laboratory-reagent grade 2-thiobarbituric acid is not necessary if the blank is treated in exactly the same way as the standard and sample solutions.

Standard sulphonamide solutions—Prepare as described[7] for sample solution A in A.O.A.C. method No. 32.286; use 0·1000 g each of pure sulphadiazine, sulphamerazine and sulphathiazole. For the determination of sulphamerazine and sulphathiazole, place 5 ml of each solution in separate 25-ml calibrated flasks, and dilute each to the mark with 0·1 N hydrochloric acid. Each of these solutions contains 10 μg of sulphonamide per ml (1 mg per 100 ml). For the determination of sulphadiazine, place 5 ml of the sulphadiazine solution in a 10-ml calibrated flask, and dilute to the mark with 0·1 N hydrochloric acid. This solution contains 25 μg of sulphadiazine per ml (2·5 mg per 100 ml).

PROCEDURE FOR SULPHADIAZINE—

Determine sulphadiazine as described in A.O.A.C. method No. 32.286,[7] but use 10-ml calibrated flasks instead of tubes fitted with ground-glass stoppers. Dilute 1-ml portions of sample and standard solutions and 0·1 N hydrochloric acid (blank) to 10 ml with buffered 2-thiobarbituric acid solution. Place the flasks in a bath of boiling water, insert the stoppers when the solutions have attained the temperature of the bath, and then heat for 1 hour. Remove the stoppers to prevent them from sticking, allow to cool, and add water, if necessary, to compensate for any loss of moisture. Continue as in the A.O.A.C. method.

PROCEDURE FOR SULPHAMERAZINE AND SULPHATHIAZOLE—

By pipette, place 5 ml of sample solution A (prepared as described[7] in A.O.A.C. method No. 32.286) in a 25-ml calibrated flask, dilute to the mark with 0·1 N hydrochloric acid, and measure the optical density of the solution at 243 and 280 mμ against 0·1 N hydrochloric acid. Determine the values of $E^{1\%}_{1\,cm}$ for the three sulphonamides from measurements made on the standard solutions containing 1 mg of sulphonamide per 100 ml. Substitute the values so obtained in the above equations, and calculate the concentrations of sulphamerazine and sulphathiazole in the sample solution. Hence deduce the content of each sulphonamide in the original sample.

Results and discussion of the method

The proposed procedure was tested on four mixtures containing known amounts of the three sulphonamides; the results are shown in Table I. A variety of commercial products, both locally prepared and imported from various countries, including tablets as well as liquid preparations (suspensions) was then analysed, and some of the results are shown in Table II.

The accuracy of the results is as good as can be expected from a simultaneous spectrophotometric procedure for mixtures. For commercial preparations, tolerances in the amounts present must naturally be added to the analytical error. Some interference occurs in the colorimetric determination of sulphadiazine when the other two sulphonamides are present in large excess, *e.g.*, 20 times the amount of sulphadiazine (see mixture No. 4 in Table I). This, however, is a proportion unlikely to be found in commercial preparations.

Table I

Sulphonamide contents found in prepared mixtures

Mixture No.	Sulphadiazine content— present, mg	Sulphadiazine content— found, mg	Sulphamerazine content— present, mg	Sulphamerazine content— found, mg	Sulphathiazole content— present, mg	Sulphathiazole content— found, mg
1	175	175	175	170	175	176
2	250	248	150	154	100	98
3	125	126	50	48	325	323
4	25	28	300	304	175	168

Table II

Sulphonamide contents found in commercial trisulphonamide preparations

The figures for samples A, C and D are expressed as milligrams of sulphonamide per tablet and those for sample B as milligrams of sulphonamide per fluid drachm (3·6 ml)

Sample	Sulphadiazine content— stated, mg	Sulphadiazine content— found, mg	Sulphamerazine content— stated, mg	Sulphamerazine content— found, mg	Sulphathiazole content— stated, mg	Sulphathiazole content— found, mg
A (tablets) ..	185	183	130	135	185	196
B (suspension)	185	184	130	127	185	186
C (tablets) ..	100	96	200	198	150	157
D (tablets) ..	185	75	130	Nil	185	301

The four commercial products tested in Table II were from three different countries. Sample D was obviously a falsification, in which the amount of sulphathiazole (the cheapest of the three sulphonamides) was double that indicated, the more expensive sulphadiazine was present in less than half the amount stated and the most expensive sulphamerazine was absent.

The proposed method is rapid and simple; it fulfils the requirements of a routine procedure for determining the three individual sulphonamides in their most common combination. Semi-skilled operators have analysed, without difficulty, a batch of four samples in duplicate during one morning. Commonly used excipients have caused no interference.

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Anhydrous Sodium Carbonate as an Acidimetric Standard

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The status of anhydrous sodium carbonate as an acidimetric standard is reviewed, and a preparative method is described that avoids the difficulties mentioned in earlier literature. The purity of the product is established by analysis.

ANHYDROUS sodium carbonate has been widely used as an acidimetric standard since the earliest recommendations by Lunge,[1,2,3] but, from time to time, doubts have been cast upon its suitability for work of the highest accuracy. Much conflicting evidence has been published about the apparent difficulty of rendering the salt completely anhydrous without causing it to lose carbon dioxide and consequently to exhibit enhanced alkalinity.[1,3 to 15] Material for analytical standardisation is commonly prepared or purified via the hydrogen carbonate or the so-called sesquicarbonate, and it is clear from an examination of the published facts that products having different purities can be obtained by utilising different conditions for the final stage of thermal conversion. There is, for example, much evidence that the presence of silica may have an adverse effect on this conversion in accordance with the reaction[5,7,15]—

$$Na_2CO_3 + SiO_2 \longrightarrow Na_2SiO_3 + CO_2$$

and it is therefore unfortunate that all workers have not specified the type of vessel used. There is also evidence that free or combined water removed at elevated temperatures may partly hydrolyse the product, with consequent formation of some free alkali; both effects, as might be expected, seem to be greater at higher temperatures. Although heating under these adverse conditions may lead to falsely high titres in the subsequent standardisation, failure to complete the removal of the water will have the reverse effect.

These findings are not inconsistent with the several reports that anhydrous sodium carbonate is stable when heated at about 700° to 800° C in platinum,[15,16,17] and claims that a satisfactory product can be obtained by heating the carbonate in a stream of carbon dioxide[6,10,15] may be equally concordant, although it is not always clear whether a vessel of platinum or some other material was used. At text-book level, it has been customary to recommend that the hydrogen carbonate or carbonate be heated at temperatures between about 260° and 300° C,[18,19] preferably in platinum. In our laboratory, such treatment of AnalaR anhydrous sodium carbonate has consistently yielded a product giving an almost theoretical titre in terms of benzoic acid that had been exhaustively purified by zone-melting (P.V.S. grade, "Purified for Volumetric Standardisation").

During work aimed at the ultimate production of anhydrous sodium carbonate for inclusion in the P.V.S. range of reagents,[20] we have prepared samples by many of the recommended procedures and variants thereof. At an early stage in this programme, the preparation of sodium sesquicarbonate was examined, mainly because this compound was used as an intermediate in the production of the working standard sodium carbonate described in the well known standardisation scheme of the Analytical Chemists' Committee of Imperial Chemical Industries Ltd.[12,14] Unfortunately, the directions for the preliminary isolation of the sesquicarbonate led to the production of material found to be mainly sodium hydrogen carbonate, and, after a repetition of this experience, it was decided to investigate alternative methods. There is little doubt that the end-product would have been satisfactory, as the stage in question would have effected some purification of the starting material, but we did not complete the conversion because the first part of the process did not seem to be the most efficient way of preparing pure specimens of the sesquicarbonate or hydrogen carbonate. Later experiments indicated that preparation of the pure sesquicarbonate was not particularly easy on a laboratory scale, and our interest in it was diminished by the results of a parallel series of experiments, which suggested that a satisfactory material would result from the ignition of purified carbonate or hydrogen carbonate in a platinum vessel. Subsequently, and because our work was directed towards the production of moderately large batches, it was thought worth-while to attempt to dispense with the use of platinum apparatus. Both

nickel and silver vessels gave rise to superficial contamination that was difficult to remove if the material had to be stirred, and it was therefore decided to try porcelain or glass vessels under conditions that might not give rise to difficulties of the kind experienced by other workers. Thus, high temperatures were to be avoided, and this consideration favoured the initial production of normal carbonate rather than hydrogen carbonate, but gave rise to the additional problem of removing most of the water of crystallisation at a very low temperature so as to prevent the hydrated carbonate from melting in contact with the porcelain or glass.

Preparation of sodium carbonate

Sodium carbonate decahydrate was therefore prepared by passing a slight excess of carbon dioxide into a 25 per cent. w/v solution of a selected batch of AnalaR sodium hydroxide in de-mineralised water. Sodium hydroxide was chosen as starting material because it was possible to select batches of this substance having lower contents of trace impurities than had the available sodium carbonate. The carbon dioxide used throughout was "cylinder gas" containing about 0·5 per cent. of air and inert gases, less than 0·000025 per cent. of sulphur, less than 0·00005 per cent. of nitrous oxide and no detectable halogen (this information was provided by the manufacturers, the Distillers Co. Ltd., Dagenham, Essex); it was passed through moist cotton-wool, as cleanser, before use.

The product was recrystallised from water and was then partly dehydrated, at room temperature, over anhydrous calcium chloride in an unvented oven fitted with an efficient fan to maintain continuous circulation of air above a large surface of the carbonate (contained in Pyrex-glass trays). When the water content had been decreased to approximately 3 moles, the temperature was slightly increased to 30° C and was maintained at this level until a further decrease in water content, down to about 1·5 moles, had been achieved; this last stage took about 30 days. Finally, the product was transferred to porcelain basins and heated at 230° C under an atmosphere of carbon dioxide until it gave a constant titre (heating for about 1 hour was necessary). The product was analysed by standard methods[21] and was found to contain less than 0·0005 per cent. each of silica, iron and magnesium *plus* alkaline earths, less than 0·001 per cent. of potassium, less than 0·0003 per cent. of chloride and less than 0·0025 per cent. of sulphate.

Attempts to devise a sensitive test for the direct determination of free alkalinity failed, but three successive batches, each of about 1 kg, were assayed by titration with gravimetrically standardised hydrochloric acid. The results confirmed that the material was suitable for standardising strong acids, within the commonly accepted limits of accuracy for purely volumetric analysis.

Analytical techniques

Treatment of pipettes—

Two bulb-pipettes of nominal capacities 10 and 50 ml were filled with Repelcote solution (a 2 per cent. solution of dimethyldichlorosilane in carbon tetrachloride), set aside for 1 minute and allowed to drain. After being allowed to "dry" in air and then washed with water and acetone, each pipette was re-calibrated by weighing the water delivered after it had been filled so that the flat meniscus coincided with the calibration mark. Repetition of these calibrations showed the "10-ml" and "50-ml" pipettes delivered 10·069 ± 0·001 ml and 50·162 ± 0·005 ml, respectively.

Standardisation of hydrochloric acid—

Stock solutions of N hydrochloric acid were prepared by diluting AnalaR concentrated acid with de-mineralised water and were stored in polythene containers. Samples (200 ml) of these solutions were maintained at 20° ± 0·1° C for 2 hours, 10·069-ml portions were placed, by pipette (at 20° C), in separate 250-ml beakers, and each was treated as described below.

A 25-ml portion of water, 1 ml of approximately 5 N nitric acid and a solution containing 2·0 g of AnalaR silver nitrate dissolved in 25 ml of water were added. The contents of the beaker were rapidly heated to boiling-point, with stirring, and then allowed to cool to about 20° C in the dark. The precipitated silver chloride was collected on a weighed porcelain Gooch crucible (padded with asbestos that had been previously extracted with hot dilute nitric acid). Transference of the precipitate was assisted with extremely dilute nitric acid, and exposure to light was minimised throughout.

After being dried to constant weight at 140° C the crucible was re-weighed. Corrections for buoyancy were applied, although they were hardly significant because of the high density of silver chloride.

Triplicate standardisations of two different batches of acid were consistent to within ±0·1 mg on weights of approximately 1·5 g of silver chloride.

ASSAY OF SODIUM CARBONATE—

Before assay, samples of the prepared sodium carbonate were heated at about 300° C for 1 hour under an atmosphere of carbon dioxide in a platinum crucible fitted with a Rose-type lid; the temperature was kept approximately constant by using a gas-heated sand-bath having a mercury thermometer in the sand. Each sample was then treated as described below.

A weight of carbonate equivalent to 0·1 ml of N hydrochloric acid less than the volume delivered by the "50-ml" pipette was weighed into a 250-ml conical flask and dissolved in 25 ml of water. The flask was connected to a reflux condenser, and 50·162 ml (at 20° C) of the standardised hydrochloric acid were added, by pipette, via the condenser. The mixture was heated under reflux for 5 minutes to expel carbon dioxide, the condenser was rinsed with distilled water, and the solution was quantitatively transferred to a 400-ml beaker and titrated with 0·2 N sodium hydroxide delivered from a 1-ml microburette to a potentiometric end-point. A Pye pH meter (model No. 605) with conventional glass - saturated-calomel electrodes was used, and the end-point was determined via the second differential. The 0·2 N sodium hydroxide was standardised potentiometrically against the standardised hydrochloric acid.

Three batches of the specially prepared sodium carbonate were assayed in this way; the results were—

Purity of batch A, %	..	..	100·00, 100·01, 99·99
Purity of batch B, %	..	..	99·99, 100·00, 100·01
Purity of batch C, %	..	..	100·01, 100·00, 100·00

We acknowledge the assistance of Mr. P. D. Jones, who carried out the potentiometric titrations.

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An Investigation of the Quinoline Phosphomolybdate Method for Determining Phosphate

Its Applicability in International Trade

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Perrin's modification of Wilson's method for determining phosphoric acid in fertilisers has been examined. After some small improvements, the method, originally intended for routine analysis, is recommended for use as a referee method in international trade.

International trade in fertilisers is increasing every year, and it is therefore important that highly accurate analytical methods should be available for use in consumer countries as well as in producer countries. If all countries used the same referee methods, these methods would sooner or later replace other methods and should therefore be rapid as well as accurate; even if each country kept its own analytical methods, the referee method to be applied in cases of dispute ought to be rapid, so as to avoid a loss of time that might be a hindrance to international trade.

For the determination of phosphoric acid (P_2O_5) in fertilisers, several methods are currently available; these methods are based on three principles.

(1) Precipitation as ammonium magnesium phosphate (gravimetric).
(2) Precipitation as ammonium phosphomolybdate (gravimetric; involves use of an empirical factor).
(3) Precipitation as quinoline phosphomolybdate; this is an improvement on the von Lorenz method, and no empirical factor is needed, as the composition of the precipitate is in accordance with the formula stated by Wilson.[1]

The first of these principles has been the basis of several so-called referee methods and the official method of the Association of Official Agricultural Chemists.[2] Preliminary separation of phosphate by means of ammonium molybdate solution is regarded as necessary in most countries and also in our opinion. When such a method is used, the differences between duplicate determinations by one analyst can sometimes be very small, but the differences between the results of two independent laboratories are great; a tolerance of 0·5 per cent. of P_2O_5 should be allowed. Further, these methods are time-consuming.

The second principle is the basis of the von Lorenz method,[3] which is used in most Western European countries. This is a splendid method, but needs special apparatus, continuous checking of reagents and apparatus and specially trained analysts. The differences between duplicate determinations by untrained analysts is less than 0·3 per cent. of P_2O_5; duplicate determinations by better trained analysts differ by less than 0·18 per cent. and by well trained analysts by less than 0·12 per cent. Differences between two or three laboratories are generally less than 0·3 per cent.

The third principle was introduced by Wilson,[1,4] and, soon after it had been published, the method was tested in a department of this research station. The results clearly showed that the method had all the drawbacks we had encountered when testing volumetric methods based on the second principle of precipitation. The reproducibility was worse than that of the von Lorenz method; for referee purposes, therefore, Wilson's volumetric method was unsatisfactory. However, Perrin subsequently proposed two gravimetric methods[5] based on the principle of Wilson's quinoline method. The method Perrin intended for routine determinations was tested, and it immediately promised to be very rapid and highly accurate; the reproducibility was even better than that by the von Lorenz method. Further investigations were therefore made in order to ascertain whether or not this method, which we have called the Perrin - Wilson method, could replace the von Lorenz method. The tolerable amounts of acids, such as hydrochloric, nitric, sulphuric and citric, and of salts, such as sodium chloride, calcium chloride, ammonium chloride, ammonium sulphate and ammonium citrate, had to be determined, as did the applicability of the method to solutions of basic slag and those containing polymerised phosphates. Otherwise we could never be sure that the method

was applicable to all types of fertilisers and some sorts of solutions used in international trade, *e.g.*, Petermann solution (alkaline ammonium citrate). Some of this work had already been done by Wilson, and in such instances we only repeated his work.

Experimental

Influence of various compounds—

In these experiments, we used an aqueous solution containing 16·00 mg of pure potassium dihydrogen orthophosphate per 10 ml; determinations were made by the proposed method.

Hydrochloric acid—When up to 30 ml of 4 N hydrochloric acid were added to 10 ml of the test solution, without neutralisation, 16·02 to 16·06 mg of P_2O_5 were found. Further addition of the acid led to low results, bleakish yellow colloidal solutions being formed.

Nitric acid—We found, as did Wilson,[1] that reasonable amounts of nitric acid could be present, but, when this acid was used for oxidising reducing substances, results were bad.

Sulphuric acid—When up to 50 ml of 3 N sulphuric acid were added to 10 ml of the test solution, without neutralisation, the amount of P_2O_5 found was 15·99 to 16·04 mg. Further additions led to low results, greenish precipitates being formed.

Sodium chloride—The addition of up to 5 g of sodium chloride to 10 ml of the test solution produced no effect other than a negligible tendency towards high results; 16·03 to 16·06 (average 16·05) mg of P_2O_5 were found.

Calcium chloride—Our results confirmed Wilson's statement[1] about the effect of lime on the determination.

Ammonium chloride—Results were low in the presence of this salt, even when less than 0·5 g was added.

Ammonium sulphate—Results were low in the presence of more than 0·5 g of this salt (equivalent to 0·1 g of ammonia-N).

Ammonium citrate—Portions of the test solution were treated with neutral ammonium citrate solution, prepared as recommended by the A.O.A.C.[2] We found that only 5 ml of this citrate solution could be safely added; in the presence of 15 ml or more, no precipitate was formed. When 20 ml each of 4 N hydrochloric acid and an alkaline citrate solution were added, a precipitate was produced, but only a few milligrams of P_2O_5 were recovered.

Citric acid—It was found that, contrary to our expectations, only moderate amounts of citric acid could safely be added to the test solution, without neutralisation. Wilson[1] found that results were low in the presence of more than 50 ml of a 2 per cent. solution of citric acid (equivalent to 1 g of the crystalline acid).

Consideration of the results of these experiments led us to conclude that (*a*) neutralisation of the solution before precipitation was not necessary (indeed, a slightly acid initial solution might even be advantageous) and (*b*) the presence of ammonia-N could lead to large errors. We distrusted the use of nitrites and did not like Wilson's suggested use of hypobromite[1] to remove ammonia. In our opinion, treatment of the solution with sodium hydroxide and then addition of sufficient hydrochloric acid to convert the hydroxide into chloride was the best way of removing ammonia-N, as the tolerance for sodium chloride was great and the slightly acid solution would counterbalance the buffering effect of salts.

Applicability of the method to basic slag—

It was decided to test the applicability of the Perrin - Wilson method to the analysis of basic slag for P_2O_5 soluble in citric acid, and nine different samples were analysed by this and the von Lorenz method. For five samples the results were in good agreement, but for the others the Perrin - Wilson method gave high results. However, after the solutions had been oxidised with potassium chlorate, results for these samples were satisfactory (see Table I).

These results suggested that, when applying the Perrin - Wilson method, great care should be taken to keep silicic acid in solution. In a paper on the preparation of solutions in which calcium and magnesium were to be determined,[6] it was stated that small amounts of silicic acid could be kept in solution after preliminary oxidation; precipitation occurred only after reducing substances had been added.

We concluded that, by oxidising the citric acid solutions of basic slag, not only were unwanted reducing substances removed, but small amounts of silicic acid were kept in solution. The use of nitric acid or potassium permanganate led to poor results, but potassium chlorate was a satisfactory oxidising agent. In this way, it was possible to avoid the addition

of large amounts of citric acid and the time-consuming removal of silicic acid; in our laboratories, silicic acid is now seldom removed quantitatively. (Wilson's general principle of keeping silicic acid in solution by means of citric acid[4] is valid; in the suggested modification, oxidation only helps the action of the citric acid already present.)

TABLE I

COMPARISON OF RESULTS FOR P_2O_5 IN BASIC SLAG

Sample No.	P_2O_5 content found by von Lorenz method			P_2O_5 content found by Perrin - Wilson method	
	Laboratory A, %	Laboratory B, %	Laboratory C, %	Without oxidation, %	After oxidation, %
41	15·52	15·49	15·52	15·63	15·44, 15·54
45	16·26	16·35	16·37	16·39	—
47	16·24	16·27	16·29	16·57	16·32, 16·41
51	15·38	15·39	15·34	15·39	—
52	15·54	15·60	15·42	15·78	15·68, 15·56
54	16·25	16·24	16·23	16·39	16·22, 16·31
56	15·46	15·33	15·43	15·43	—
58	15·42	15·28	15·41	15·34	—
60	15·52	15·46	15·42	15·49	—

APPLICABILITY OF THE METHOD TO METAPHOSPHATES—

In these experiments, a sample of commercially available hexametaphosphate reported to contain about 67 per cent. of P_2O_5 was used. For the determination of water-soluble P_2O_5, 1·5 g of the sample were rotated for 30 minutes with 450 ml of water in a 500-ml calibrated flask, and the contents of the flask were subsequently diluted to the mark and well mixed; 10-ml portions of the solution were used for the determinations. The contents found by the von Lorenz method, in which all soluble metaphosphates are converted into orthophosphates, were 67·04 and 67·06 (mean 67·05) per cent.

When the unmodified Perrin - Wilson method was used the results were too low, *viz.*, 66·44 and 65·60 per cent. of P_2O_5. We then tried to convert the metaphosphates into orthophosphates by adding to 10 ml of the hexametaphosphate solution 1 ml each of concentrated hydrochloric acid and a solution containing 25 mg of potassium chlorate per ml and then heating the mixture in boiling water for 30 minutes. After this treatment, the results were slightly better (66·29 and 66·32 per cent. of P_2O_5). However, when the sample solution was treated as described above, but the 30-minute heating period was replaced by boiling for 3 minutes the results were 67·01 and 67·03 per cent. of P_2O_5; prolonged boiling did not lead to higher results.

TABLE II

COMPARISON OF RESULTS FOR P_2O_5 IN DOUBLE AND TRIPLE SUPERPHOSPHATES

Solutions for analysis were prepared by rotating the samples for 30 minutes with 500 ml of water

Sample No.	P_2O_5 content found by von Lorenz method on 2·5-g sample, %	P_2O_5 content found by Perrin - Wilson method on—		
		2·5-g sample, %	5-g sample, %	10-g sample, %
8	45·3	45·03	44·81	44·77
43	45·9	45·61	45·43	45·40
167	42·5	42·43	42·29	42·24
184	39·8	39·63	39·63	39·68
202	41·2	40·96	—	40·95

It was therefore concluded that an extent of conversion of metaphosphates into orthophosphates equal to that occurring in the von Lorenz method could be achieved by adding hydrochloric acid and then boiling the solution for a few minutes. Probably, this modification should also be made when the Perrin - Wilson method is applied to triple superphosphates containing more than 42 per cent. of P_2O_5 (see Table II).

Although it is known that the von Lorenz method tends to give slightly high results when applied to high-grade phosphates, the differences between results by the two methods are striking. Probably, they are caused by the presence of polymerised phosphates, or metaphosphates, which are not converted into orthophosphates by the Perrin - Wilson method. Further, the results in Table II clearly show that, for triple superphosphates containing more than 42 per cent. of P_2O_5, 2·5 g of sample should be rotated with 500 ml of water; it is not advisable to continue rotation for much longer than 30 minutes because of the risk that dicalcium phosphate might be formed and precipitated, especially when the percentage of free phosphoric acid is small.

Method

Reagents—

All solutions should be prepared in distilled or de-mineralised water. The molybdate reagent and quinoline solutions are the same as those used by Perrin.[5]

Molybdate reagent solution—Place 54 g of analytical-reagent grade molybdenum trioxide in a 500-ml beaker, add 200 ml of water and then 11 g of analytical-reagent grade sodium hydroxide, with stirring, and warm until solution is complete. Place 60 g of analytical-reagent grade crystalline citric acid in a 1-litre beaker, dissolve in 250-ml of water, and add 140 ml of hydrochloric acid, sp.gr. 1·18. With effective and constant stirring, pour the molybdate solution into the citric acid solution, cool, filter the solution into a 1-litre calibrated flask, and dilute to the mark with water. Store the solution in the dark in a well stoppered polyethylene bottle.

Quinoline solution—Place 60 ml of hydrochloric acid, sp.gr. 1·18, in a 1-litre beaker, dilute with 300 or 400 ml of distilled water, and heat to 70° or 80° C. Pour 50 ml of synthetic quinoline (of the highest grade of purity obtainable and free from reducing agents) in a thin stream and with constant stirring into the diluted acid. When the quinoline has dissolved, cool, dilute with water, filter into a 1-litre calibrated flask, and dilute to the mark. Store the solution in a polyethylene bottle.

Hydrochloric acid, sp. gr. 1·18.

Hydrochloric acid, 3 N.

Sodium hydroxide, 3 N.

Potassium chlorate solution—Dissolve 2·5 g of potassium chlorate in water, and dilute to 100 ml.

Treatment of sample solution—

With a graduated pipette, transfer an aliquot of the sample solution containing not more than 25 mg of P_2O_5 (11 mg of P) to a 250-ml beaker.

Samples containing ammonia-N—If the aliquot taken contains more than 100 mg of ammonia-N, add, for each 40 mg of ammonia-N present, 1 ml of 3 N sodium hydroxide, and then add 25 ml of water. Boil for about 15 minutes, cool, slowly add an equivalent amount of 3 N hydrochloric acid and then 1 ml in excess, and continue as described under "Determination of Phosphate."

Samples containing ammonium citrate—If the aliquot taken contains neutral (*e.g.*, solution from A.O.A.C. method) or alkaline ammonium citrate (*e.g.*, Petermann solution), add, for each millilitre of the original citrate solution present, 1 ml of 3 N sodium hydroxide. Boil for 15 minutes, add the same amount of 3 N hydrochloric acid to convert all sodium hydroxide into sodium chloride, and then add 1 ml of acid in excess. Continue as described under "Determination of Phosphate."

Samples containing reducing substances—If the aliquot taken contains reducing substances, as with citric acid solutions of basic slag, add 1 ml each of hydrochloric acid, sp.gr. 1·18, and the potassium chlorate solution. Boil for 3 minutes, and continue as described under "Determination of Phosphate."

Samples containing metaphosphates etc.—If the aliquot taken contains metaphosphates or polymerised phosphates, or both, add 1 ml of hydrochloric acid, sp.gr. 1·18, boil for at least 3 minutes, and continue as described under "Determination of Phosphate." It should be borne in mind that solutions in water, citric acid or citrates sometimes contain such phosphates, especially if the sample contains more than 42 per cent. of P_2O_5.

DETERMINATION OF PHOSPHATE—

Dilute the solution to about 110 ml with water, add 25 ml of molybdate reagent solution, heat to boiling-point, and place the beaker in a bath of boiling water for 15 minutes (no precipitate should be formed at this stage). Add 12·5 ml of quinoline solution from a burette; in order to produce a precipitate having maximum particle-size, the first few millilitres of quinoline solution should be added dropwise and the rest in a thin stream, with constant swirling of the beaker. When the quinoline solution has been added, shake the beaker for 3 minutes.

Cool the beaker and its contents under a stream of running tap-water to room temperature (20° ± 1° C), and separate the precipitate on a glass or porcelain crucible having pores 5 to 15 μ in diameter; the crucible should have been previously dried for 15 minutes at 250° C, allowed to cool in a desiccator and then weighed. Transfer the last traces of precipitate from the beaker to the crucible with a thin stream of water from a wash-bottle, and finally wash the precipitate with water. Dry the crucible and precipitate for 15 minutes at 250° C, allow to cool in a desiccator, and weigh. Convert the weight of precipitate to weight of P_2O_5 by multiplying by the factor 0·03207 or to weight of P by multiplying by 0·01401.

DISCUSSION OF THE METHOD

The Perrin - Wilson method is an excellent technique and could be used as a referee method. However, certain modifications are necessary when analysing basic slag and solutions containing metaphosphates or polymerised phosphates and also when much ammonia-N is present. Further, any neutralisation of acid solutions can be omitted, and it is even advisable to begin with a slightly acid solution.

Comparison between the Perrin - Wilson and von Lorenz methods led us to the conclusions listed below.

(1) With the von Lorenz method, one analyst can handle 40 to 50 samples per day, as against 20 to 30 samples with the Perrin - Wilson method. However, in the von Lorenz method, it is advisable to set the precipitate aside overnight (or at least for 2 hours), and this is not necessary in the Perrin - Wilson method; often, therefore, results are available 1 day earlier when the Perrin - Wilson method is used.

(2) The tolerance for ammonia-N is at least five times greater in the von Lorenz method than in the Perrin - Wilson method.

(3) The tolerance for sulphuric acid is twice as great in the Perrin - Wilson method as it is in the von Lorenz method.

(4) When metaphosphates are present, special precautions are necessary in the Perrin - Wilson method, but not in the von Lorenz method.

(5) The reproducibility of results is better by the Perrin - Wilson method than by the von Lorenz method.

We therefore suggest that the Perrin - Wilson method, with the proposed modifications, should be adopted as a referee method in international trade.

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A Turbidimetric Method for Determining Elemental Sulphur

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A method is proposed for determining elemental sulphur by solution in acetone and then precipitation by exchange of solvent. The optical density of the colloidal solution so produced has been shown to bear a linear relationship to the concentration of sulphur between 1 and 7 μg per ml. It is suggested that the method would be suitable for determining elemental sulphur present in soil to an extent greater than 1 mg per g of dry material.

THE presence of elemental sulphur in tidal-swamp soils may hinder their reclamation by empoldering and drainage. In deposits low in calcium, oxidation of reduced sulphur compounds can lead to the development of excessive soil acidity. During an investigation of the part played by sulphur-oxidising bacteria in producing acid conditions in such soils, an attempt was made to evolve a rapid method for determining elemental sulphur, since this substance is likely to be the most readily oxidised by these organisms.

The only direct method known to me was that described by Ory, Warren and Williams,[1] who utilised the colour reaction between sulphur and Schönberg's reagent, benzyliminodi-(*p*-methoxyphenyl)methane, as the basis of a colorimetric determination. As originally described, the method was not adapted to the analysis of soils and would be tedious as a routine method. It would involve extraction of the soil with a suitable solvent and evaporation of the extract to dryness before the colour reaction could be carried out. The method is relatively insensitive, requiring from 1 to 22 mg of sulphur in 25 ml of final solution, and was not shown to be free from interference by organic matter extracted by the solvent from the soil.

This paper reports the results of an attempt to devise a method based on the direct precipitation of colloidal sulphur from a soil extract by solvent exchange.

Experimental

General—

Smittenburg, Harmsen, Quispel and Otzen,[2] in their reductive determination of sulphur compounds in soil, used acetone as extracting solvent. In this investigation, satisfactory colloidal solutions were obtained with acetone or ethanol, but, as the solubility of sulphur in ethanol is less than in acetone (about 500 μg of sulphur per ml of acetone at 25° C), the latter solvent was adopted. Solution of sulphur in the acetone occurs almost instantaneously, and it has been found that, for finely ground soil, extraction for 5 minutes is adequate for the removal of the elemental sulphur present. The period of extraction was kept as short as possible to minimise solution of organic material, which might interfere with formation of the colloidal solution of sulphur.

The light-absorbing properties of the solution increase as the wavelength of the light used decreases, and a wavelength of 420 mμ was found to give consistent results in the absence of colour in the acetone extracts. The rate at which the optical density of the colloidal solution developed was found to depend on the way in which the acetone and water were mixed. If water was added to the acetone extract, maximum absorption developed in 2 hours, but the rate of addition had to be carefully standardised; in practice, this method led to less consistent results than did the one proposed. The change in optical density with time when the colloidal solution of sulphur was prepared by the proposed method is shown in Fig. 1. The solution is stable for 30 minutes at maximum absorption.

The optical density is to some extent decreased at low temperatures, but the decrease produced over the range of temperatures encountered in this laboratory (32° to 20° C) was insignificant. Experiments on the proportions of water and acetone in the final mixture showed that maximum absorption was unaffected by concentrations of acetone up to 20 per cent.

CALIBRATION—

The calibration graph shown in Fig. 2 was plotted from the results obtained when aliquots of a standard solution containing 156·8 mg of sulphur per 500 ml of acetone were used. (Standard solutions were preserved for future use in 50-ml calibrated flasks, so that losses by evaporation could be corrected.) This graph is typical of several obtained by using different solutions. The relationship between concentration of sulphur present and optical density approaches linearity over the range shown. At higher concentrations of sulphur the absorption does not increase proportionately.

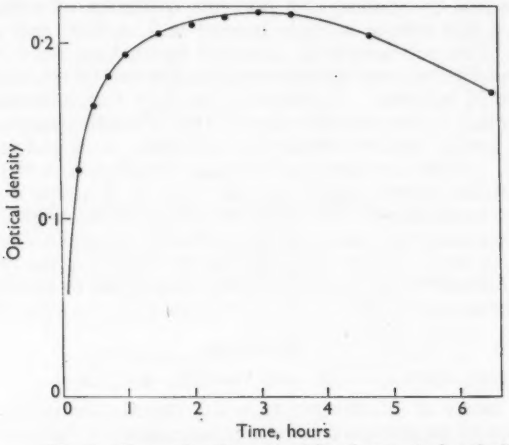

Fig. 1. Curve showing rate of development of optical density of a colloidal solution of sulphur prepared by adding a solution of sulphur in acetone to distilled water. Each point on the curve represents the mean of three measurements

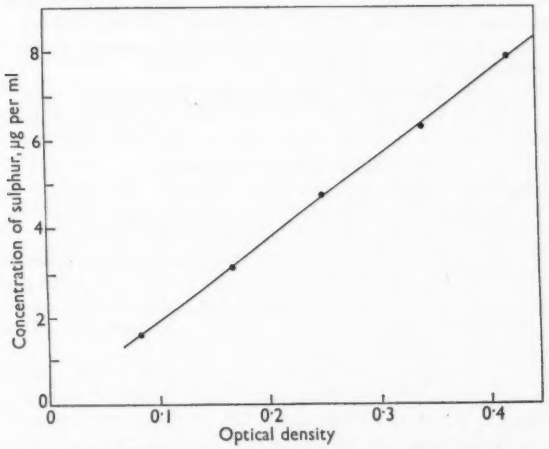

Fig. 2. Relationship between optical density and concentration of sulphur. Each point on the curve represents the mean of duplicate measurements

METHOD

APPARATUS—

Centrifugation was carried out in 100-ml tubes in an M.S.E. soil centrifuge (Measuring and Scientific Equipment Ltd.).

Optical-density measurements were made in matched tubes (diameter 45 mm) with a Unicam SP400 spectrophotometer.

REAGENTS—

Standard solutions of sulphur were prepared in analytical-reagent grade acetone, which was also used for extracting the soil. Recrystallised sulphur was used throughout.

PROCEDURE—

The sample of soil is dried under vacuum over phosphorus pentoxide in the presence of potassium hydroxide and pyrogallol; this prevents oxidation of sulphur before the determination. When dry, the sample is finely ground in a mortar, and a small sub-sample is accurately weighed. This sub-sample is extracted by shaking for 5 minutes with acetone in a centrifuge tube, and a clear supernatant liquid is obtained by centrifugation at 2000 r.p.m. and 22-cm radius for 15 minutes. A suitable aliquot of this solution, containing between 100 and 700 μg of sulphur, is then run into about 80 ml of distilled water in a 100-ml calibrated flask, the flask being gently agitated during the addition. Colloidal sulphur begins to form immediately after the solvent has been exchanged. The liquid in the flask is then diluted to the mark with distilled water, mixed and set aside for 3 hours, after which the optical density of the solution is measured. The sulphur content of the original sample is calculated by reference to a calibration graph plotted from the results obtained when a standard solution of sulphur in acetone is used. If the concentration of sulphur in the extract exceeds 200 μg per ml, the extraction should be repeated at a lower ratio of soil to acetone to ensure complete removal of sulphur from the sample.

RESULTS

RECOVERY TESTS IN PRESENCE OF SOIL AND ORGANIC MATTER—

The most likely source of interference to which the method might be susceptible when used in soil analysis would be acetone-soluble organic matter. This might be co-precipitated with the sulphur or otherwise affect formation of the colloidal solution of sulphur. It is at once obvious that the method would therefore be unsuitable for determining sulphur in soil on the micro scale and might also suffer from significant interference if used with peaty soils yielding coloured extracts. However, the sulphur-rich soils of tidal areas may contain up to 50 mg of oxidisable sulphur per g of dry matter; as much as half of this sulphur might be in the elemental form, according to the prevailing soil conditions. With such soils, no trouble from interfering organic matter need be expected, owing to the small amount of soil needed to produce measurable absorption.

In order to determine the approximate sulphur content at which interference would become significant, a series of recovery tests was carried out in which standard solutions of sulphur were shaken for the normal extraction period with different weights of soil and also with samples of the organic matter likely to be found in the particular soils for which the method was designed. Eight soils having different contents of organic matter were examined, but it was not possible to include samples of mangrove soils, as these invariably contain significant amounts of sulphur, and no sufficiently accurate method was available for comparison. For this reason, the samples of organic matter used were (*a*) fibrous rootlets of the mangrove *Rhizophora racemosa*, which accumulate in soils colonised by this mangrove, (*b*) rice straw, (*c*) rice roots and (*d*) material from the grass *Paspalum vaginatum*, a common weed on reclaimed mangrove land in West Africa. All samples of organic matter were dried in air and ground in a hammer mill before use.

Aliquots (5 ml) of a standard solution of sulphur and 20-ml portions of acetone were shaken for 5 minutes with 0·5-, 1·0- and 2·0-g samples of the eight soils and with 0·5-g samples of the organic materials. After centrifugation, the extracts were analysed by the proposed method, 5-ml aliquots being used for precipitation; control analyses were carried out on the standard solution at the same time. The results are shown in Table I, from which it can be seen that recoveries were satisfactory from all the soils except at the lowest concentration of sulphur, when some results were erratic. There was no relationship between the consistency of recovery and the carbon content of the soil as measured by the Walkley - Black method. *Rhizophora* rootlets, the major source of organic matter in the soils studied, had no significant effect on the recovery; neither had rice straw. The presence of material from *Paspalum* and rice roots, both of which gave coloured extracts, resulted in high recoveries.

Table I

Recovery of elemental sulphur in presence of soil and organic material

Sample No.	Description of sample	Carbon content of soil,* %	Recovery of sulphur when approximate amount added was— 3·0 mg per g of dry sample, %	1·5 mg per g of dry sample, %	0·7 mg per g of dry sample, %
1	Ferrialitic soil	0·3	100·1	99·0	113·1
2	Ferrialitic soil	1·3	105·1	101·9	100·0
3	Ferrialitic soil	2·7	102·3	102·9	103·0
4	Ferrialitic soil	4·0	98·9	105·3	104·2
5	Garden loam	3·3	96·5	97·6	99·4
6	Potting soil	5·6	100·0	103·4	113·1
7	Hydromorphic soil	1·5	100·4	100·0	97·6
8	Hydromorphic soil	3·4	96·1	99·0	94·0
9	*Rhizophora* rootlets	—	97·7	—	—
10	Rice straw	—	100·0	—	—
11	Rice roots	—	124·4	—	—
12	*Paspalum*	—	118·1	—	—

* Determined by the Walkley - Black method.

Table II

Replication obtained in analysis of tidal mangrove soils

Each sub-sample was extracted with 25 ml of acetone

Sub-sample No.	Optical density*	Mean optical density for soil	Sulphur content, mg per g of dry soil
Soil A; 0·5-*g sub-samples*—			
1	0·230, 0·244, 0·234 (mean 0·236)	0·238	4·40
2	0·235, 0·240, 0·241 (mean 0·239)		
3	0·240, 0·235, 0·238 (mean 0·238)		
Soil B; 1·0-*g sub-samples*—			
1	0·330, 0·320, 0·320 (mean 0·323)	0·317	2·95
2	0·299, 0·297, 0·308 (mean 0·301)		
3	0·325, 0·330, 0·323 (mean 0·326)		

* Each result is for a separate determination.

Reproducibility—

Table II shows the results obtained when the proposed method was used to determine the elemental-sulphur contents of two typical tidal mangrove soils. It can be seen that the reproducibility of the results for replicate sub-samples and replicate solutions prepared from the same extract is satisfactory.

Discussion of the method

The proposed method is over twenty times as sensitive as the thioketone method mentioned earlier[1] and, in the complete absence of interfering organic matter, *e.g.*, in inorganic mixtures, would be suitable for determining microgram amounts of elemental sulphur.

When applied to soil analysis, the method is likely to be subject to interference from acetone-soluble organic matter, but the results of the recovery tests suggest that such interference will not be significant when the sulphur content being determined is greater than 1 mg per g of soil. However, the method should not be applied uncritically to soils having a high content of soluble organic matter. It is expected that its main application will be in the study of the sulphur-rich soils of coastal areas, such as tidal mangrove soils, in which an elemental-sulphur content of 1 mg per g is likely to be near the lower limit. If the method were to be used for soils containing only microgram amounts of sulphur, a careful study of the interferences involved would be necessary.

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Notes

THE DETERMINATION OF FENCHLORPHOS RESIDUES IN MILK

THE insecticide *OO*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate, which now has the common name fenchlorphos, is marketed under names such as Etrolene, Nankor and Korlan. Its primary use is on cattle and sheep against a number of internal and external parasites. Residues of fenchlorphos have been detected in the milk from cattle treated orally or dermally with large doses of the insecticide.

The method here described for determining residues of fenchlorphos in milk is based on Jones and Riddick's acetonitrile - hexane partition[1] and Laws and Webley's general method for determining insecticide residues.[2] The lower limit of detection is set by the value of the blank, which by this method is the same for a 200-g sample of milk as for a 50-g sample and is equivalent to 5 μg of the insecticide; this corresponds to 0·025 p.p.m. in a 200-g sample and 0·10 p.p.m. in a 50-g sample. The choice of sample size will therefore be determined by the magnitude of residue expected. The method for a 200-g sample is described, as the procedure for the smaller sample is identical, the volumes of solvents used up to the beginning of the chromatographic stage being proportionately decreased. From the chromatographic stage onwards, the amounts of reagents used are the same for both weights of sample.

APPARATUS—

Absorption spectrophotometer.

Centrifuge.

Glass tubes for chromatography—Tubes 1·5 cm in diameter and 14 cm long, fitted with a tap at the lower end and a B19 ground-glass joint at the upper end for attachment to a reservoir of solvent.

REAGENTS—

All reagents must be of recognised analytical grade and phosphate-free.

Acetonitrile.

Dichloromethane.

Light petroleum, boiling range 40° to 60° C.

Methanol, absolute.

Alumina—Heat chromatographic aluminium oxide at 500° C for 2 hours to ensure conversion to the γ-form. Adjust the activity to Brockmann grade III by adding 6·5 per cent. w/w of water.

Perchloric acid, 1 N.

Sulphuric acid, 10 *and* 1 N.

Nitric acid, sp.gr. 1·420.

Hydrochloric acid, sp.gr. 1·180.

Ammonia solution, sp.gr. 0·880.

Ammonium molybdate solution—Dissolve 50 g of ammonium molybdate in 400 ml of 10 N sulphuric acid, and dilute to 1 litre with distilled water.

Stannous chloride solution, concentrated—Dissolve 10 g of stannous chloride dihydrate in 25 ml of hydrochloric acid, sp.gr. 1·180.

Stannous chloride solution, dilute—Dilute the concentrated solution 200-fold with 1 N sulphuric acid. Prepare a fresh solution daily.

Ethanolic sulphuric acid—Mix 5 ml of concentrated sulphuric acid with 245 ml of absolute ethanol.

Isobutyl alcohol - benzene mixture, (1 + 1, *v*/*v*).

Standard fenchlorphos solution.

Potassium dihydrogen orthophosphate.

PROCEDURE—

Transfer 200 g of the thoroughly mixed sample of milk to a 1-litre separating funnel. If an addition of insecticide is required, as for the preparation of a standard graph, add an aliquot of the insecticide solution, mix, and set aside for 5 to 10 minutes (some comments on the time of standing are made later). Add 200 ml of methanol, mix, and then add 200 ml of dichloromethane. Shake the funnel vigorously for not less than 1 minute, transfer its contents to suitable centrifuge bottles, and spin in a centrifuge at 2500 r.p.m. and 20-cm radius for 15 minutes. Return the mixture, which consists of aqueous, organic and semi-solid layers, to the funnel, and separate the

organic lowest layer. Re-extract the aqueous and semi-solid layers with a further 200 ml of dichloromethane, spin in the centrifuge, and separate as before. Combine the two extracts, and distil off the dichloromethane at atmospheric pressure; remove the last traces of solvent by blowing a current of air across the surface of the evaporating liquid. To avoid losses, it is important that the flask should not be heated overmuch in removing the last traces of solvent and that heating should cease as soon as all the solvent has evaporated. No attempt should be made to remove the trace of methanol remaining in the fatty residue after evaporation of the dichloromethane.

Dissolve the fatty residue in 50 ml of light petroleum, and transfer the solution to a 500-ml separating funnel. Add 50 ml of acetonitrile, shake vigorously for 1 minute, and run the lower acetonitrile layer into a 500-ml conical flask. Add a further 50 ml of acetonitrile to the contents of the separating funnel, extract, and separate as before. Discard the extracted light petroleum solution, and return the combined acetonitrile extracts to the funnel. Rinse the conical flask with 50 ml of light petroleum, add the rinsings to the contents of the funnel, and shake. Run the acetonitrile into the conical flask, wash the light petroleum with 15 ml of acetonitrile, and run the acetonitrile layer into the flask. Discard the light petroleum, rinse the funnel with light petroleum, and discard the rinsings.

Return the combined acetonitrile extracts (115 ml) to the separating funnel, add 250 ml of a 1·5 per cent. solution of sodium chloride, and mix thoroughly. Rinse the flask with 100 ml of light petroleum, add the rinsings to the contents of the funnel, and shake vigorously. (It may happen that, through the use of too much acetonitrile or too concentrated a solution of sodium chloride, the mixture will separate into three layers instead of two; in this event, add more water, with shaking, until only two layers are apparent.) Run the aqueous acetonitrile layer into the flask, and decant the light petroleum extract into a suitable distillation flask. Return the aqueous solution to the funnel, and similarly extract it twice more with 100-ml portions of light petroleum. Discard the aqueous acetonitrile solution, and evaporate the combined light petroleum extracts to 10 to 15 ml.

Prepare a column 1·5 cm in diameter from 8 g of the Brockmann grade III alumina; use light petroleum as liquid phase. Place a layer (about 5 mm deep) of granular anhydrous sodium sulphate on the top of the column, and add a little sodium sulphate to the light petroleum solution if this contains droplets of water. Transfer the extract to the column, elute with 100 ml of light petroleum at a rate not exceeding 2 ml per minute, and collect the eluate in a 150-ml conical flask fitted with a ground-glass joint.

Evaporate the eluate almost to dryness by heating on a water bath; assist evaporation with a current of air. Add 8 ml of water and then 4 ml of N perchloric acid, 1 ml of hydrochloric acid, sp.gr. 1·180, and 5 ml of nitric acid, sp.gr. 1·420, and wash the walls of the flask with 2 to 3 ml of water. Blow away any residual light petroleum, and heat the solution under reflux beneath a Liebig condenser for about 40 minutes. Drain the cooling water from the condenser, and continue to heat until brown fumes begin to appear. At this point, remove the condenser, and evaporate the solution until white fumes of perchloric acid are evolved. Rinse the condenser with 6 to 7 ml of water, add the rinsings to the contents of the flask, and again evaporate until white fumes are evolved. Add 3 ml of water and 4 ml of ammonia solution, sp.gr. 0·880, and remove the excess of ammonia by boiling. From this point, apply Berenblum and Chain's method,[3] as modified by Laws and Webley,[2] for determining orthophosphate; use potassium dihydrogen orthophosphate as primary standard.

TABLE I

RECOVERY OF FENCHLORPHOS ADDED TO MILK

200-g sample		50-g sample	
Insecticide added, p.p.m.	Insecticide recovered, p.p.m.	Insecticide added, p.p.m.	Insecticide recovered, p.p.m.
0·030	0·026	0·23	0·20
0·045	0·035	0·46	0·34
0·061	0·035	0·70	0·60
0·091	0·070	0·93	0·71
0·121	0·120	1·17	0·94
0·243	0·193	1·86	1·58
		2·33	1·82
		2·79	2·33
		3·50	2·66

Results

The results of some recovery experiments are shown in Table I. It was found that both 50- and 200-g samples of insecticide-free milk gave identical blank values, equivalent to 5·19 μg of fenchlorphos. This blank has been deducted in the calculation of the figures for insecticide recovered; the mean recovery was 80 per cent. for both weights of sample.

Krueger, Casida and Niedermeier[4] have referred to poor recovery of the pesticide coumaphos (Co-ral) from milk, owing to protein-binding of the organo-phosphorus molecule on standing; recovery decreased as the time of standing increased. However, although in our experiments the milk has been left for 10 minutes between the addition of the insecticide and extraction, this time is not critical. The recoveries after the treated milk had been set aside for 2 hours at this stage were the same, and even after 22 hours they were lower by only 5 to 10 per cent., irrespective of whether the samples were kept at room temperature or in a refrigerator.

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A MODIFIED PROCEDURE FOR THE COLORIMETRIC DETERMINATION OF PHOSPHORUS APPLIED TO ORGANO-PHOSPHORUS INSECTICIDE RESIDUES

During an investigation into the determination of residues of demeton-methyl in fruit and vegetables by the method described by Laws and Webley,[1] it was observed that the procedure for determining phosphorus in the final stage of the analysis was capable of producing erratic results. The technique described by these workers involves the development of molybdenum blue and is based on the method described by Berenblum and Chain[2] (as modified by Martin and Doty[3]). The source of error was traced to the partial extraction of the molybdenum-blue complex into the aqueous phase when the isobutanol - benzene solution of molybdophosphoric acid is reduced by shaking with the acid solution of stannous chloride.

In our experience, serious errors may occur, depending on the time during which the two phases are shaken together. Our experiments indicate that, if the time of shaking is extended to 30 seconds, the molybdenum blue may be almost completely extracted into the aqueous phase.

The amended procedure described below is proposed, in which the reduction is carried out in one phase, thereby eliminating this source of error. The reagents are the same as those used by Laws and Webley, with the exception that the stannous chloride solution is replaced by a freshly prepared 0·2 per cent. solution of stannous chloride ($SnCl_2.2H_2O$) in absolute ethanol.

Especial care must be taken to ensure that all reagents are satisfactorily phosphate-free. Blanks should be carried out on each new batch of reagent before it is taken into use.

Procedure—

Neutralise the phosphate solution after destruction of organic matter, and transfer to a 50-ml separating funnel. Add 3 ml of ammonium molybdate reagent solution and distilled water to make the total volume to approximately 13 ml. Add 9 ml of isobutanol - benzene mixture, insert the stopper in the separating funnel, and shake vigorously for 30 seconds. Allow the layers to separate, and discard the lower aqueous layer. Wash the remaining organic layer with 5 ml of N sulphuric acid, and again discard the lower aqueous layer. Transfer the organic layer to a stoppered 10-ml graduated cylinder, and make the volume up to 9·6 ml by the addition of ethanolic sulphuric acid. Mix, and add 0·4 ml of ethanolic stannous chloride solution. Mix thoroughly,

and measure the optical density of the solution at 730 mμ in a 1-cm cell against a blank solution of isobutanol - benzene mixture treated in the same way.

The colour development is instantaneous and the colour is stable for at least 24 hours.

A curve showing the relationship between optical density and micrograms of phosphorus present is shown in Fig. 1.

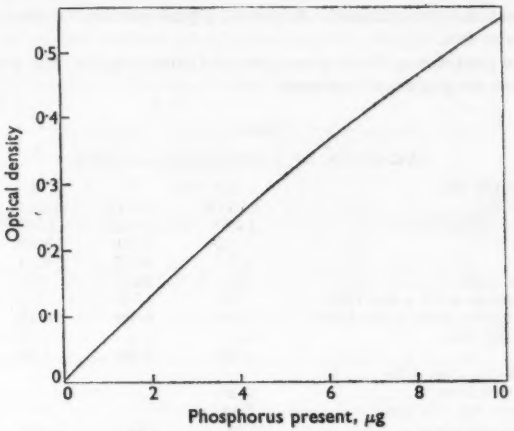

Fig. 1. Relationship between optical density and amount of phosphorus present

Since this Note was prepared, a further paper by Laws and Webley[4] has been published in which the phenomenon described above is recognised, and a maximum shaking time of 10 seconds is now recommended at the reduction stage.

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AN INVESTIGATION INTO THE PRESENCE OF ACROLEIN IN A RHINE WINE LIEBFRAUMILCH 1959

THE contents of one of five halb-stücks, each containing 600 litres of Liebfraumilch 1959, had a pungent taste; the wine was unusable. The 3000 litres were stated to have been taken from the same vat.

The Liebfraumilch was distilled, and it was found that the lower-boiling fraction contained the substance having the pungent taste. The distillate also contained ethanol, sulphurous acid, acetic acid and acetaldehyde. Tasting trials indicated that a dilute solution of acrolein had a pungency similar to that of the Liebfraumilch.

For control purposes, a solution of acrolein was prepared by dehydration of glycerol. The acrolein content was measured by forming the acrolein - bisulphite complex at pH 2·0, removing the uncombined sulphite and saturating the acrolein with iodine. The acrolein - bisulphite complex was decomposed at pH 9·5 and the sulphite determined; the solution contained 700 p.p.m. of acrolein.

The aldehyde content of the pungent wine was 264 p.p.m., as acetaldehyde, compared with 244 p.p.m. in the perfect wine (from halb-stück No. 5). German wines are high in aldehydes,

e.g., a Zeltinger Schwarzlay 1953 contained 220 p.p.m. and a Niersteiner Domthal 1957 242 p.p.m., compared with a Burgundy (Puligny-Montrachet 1959) at 84 p.p.m.

In the presence of acetaldehyde and ethanol, the acrolein was determined by adding 2 ml of 6 per cent. hydrogen peroxide to 50 ml of the wine to oxidise the sulphur dioxide and then distilling 5 ml. A direct titration was made with acidified 0·1 N bromate - bromide mixture, methyl orange being used as redox indicator. Another 5 ml of distillate were brominated for 15 minutes; there was no interference from ethanol. A second 10-ml portion of distillate from the pungent wine was free from acrolein.

Table I shows the analyses of the five samples of Liebfraumilch; the pungent wine, from halb-stück No. 1, contained 14 p.p.m. of acrolein.

TABLE I

ANALYSES OF LIEBFRAUMILCH 1959

Sample from halb-stück No.	1	2	3	4	5
Specific gravity (60° F)	1·0119	1·0118	1·0110	1·0119	1·0115
Proof spirit, %	14·64	14·78	15·04	14·64	14·60
Ethanol content, % v/v	8·42	8·50	8·66	8·42	8·50
Extract, g per litre	65·7	65·5	63·4	65·7	64·4
Sugar content, g per litre	35·5	34·8	33·6	35·3	34·3
Total acidity, as tartaric acid, g per litre	7·3	7·3	7·3	7·1	7·1
Volatile acidity, as acetic acid, g per litre	0·40	0·40	0·40	0·40	0·36
Glycerol content, g per litre	5·3	—	—	—	—
pH	3·30	3·40	3·30	3·30	3·38
Carbon dioxide content, g per litre	—	—	—	—	0·32
Nitrogen content, g per litre	0·30	—	—	—	—
Acetaldehyde content, mg per litre	264	—	—	—	244
Acrolein content, mg per litre	14	Nil	Nil	Nil	Nil
Iron content, mg per litre	7	6	7	7	7
Total-ester content, mg per litre	280	—	—	—	290
Free sulphur dioxide content, mg per litre	54	51	51	54	56
Total sulphur dioxide content, mg per litre	442	448	435	454	448

The 1959 Hocks and Moselles are lower in ethanol than the 1957 vintage by 0·5 to 1·0 per cent. v/v, and the sugar content of many is twice that found in the 1957 vintage. The wines have to be filtered into sterile casks to reduce the risk of secondary fermentation in transit from the Rhine.

Rhine wines contain, on average, 5 g of glycerol per litre, and the 2-inch-thick oak staves of the halb-stücks become saturated with glycerol when the residual wine dries out. A possible origin of acrolein is the reaction between the glycerol-impregnated oak and burning sulphur. The sulphur candles used to sterilise empty casks can drop large pieces of burning sulphur.

All the wines tested were free from *Bacillus amaracrylus*, which can cause acrylic fermentation of glycerol.

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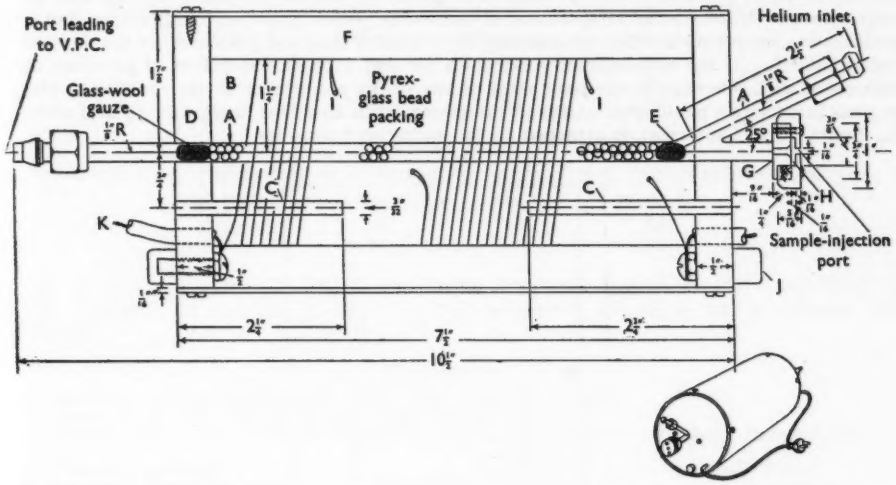
A RAPID METHOD OF QUALITATIVE AND QUANTITATIVE ANALYSIS OF PRODUCTS FROM PYROLYSIS

IN a study of the effects of structure on the pyrolysis of esters, a direct method was developed for determining the extent of decomposition and the products from pyrolysis of many compounds. The products from the pyrolysis of micro amounts of organic material were analysed directly by a gas-chromatographic column attached to a temperature-controlled pyrolysis reactor. Since the method proved particularly suited to a qualitative and quantitative study of pyrolysis products from small amounts (2 to 10 mg) of liquids (or solids that can be dissolved in suitable solvents), it is submitted as a general method for studying the pyrolysis of esters or of other compounds with similar properties. The method is accurate (error ± 2 per cent.) and requires only a few minutes for each test.

Gas chromatography has been successfully applied to a study of the products in the pyrolysis of esters in a two-stage process,[1] but has not been reported as a one-step procedure. Gas chromatography, coupled with pyrolysis techniques, has been applied to the analysis of polymers.[2] Strassburger, Brauer, Tryon and Forziati's method[3] was, however, not suited to rapid introduction of the reactant or careful temperature control. In the methods referred to, losses owing to distillation before the decomposition do not allow quantitative determinations to be made. The novelty of the method described is in the combined pyrolysis and analysis (qualitative and quantitative) and the particularly convenient method of introducing the sample.



A = Stainless-steel tube
B = Aluminium block
C = Thermocouple well
D = Transite
E = Silver-soldered joint
F = Asbestos insulation
G = Weld
H = Silicone-rubber septum strip
I = Nichrome-wire heating coil
J = Lavite cap

Fig. 1. Pyrolysis tube and thermostat

Method

Apparatus and reaction conditions—

The apparatus consists of a gas-chromatographic column and detector coupled with a temperature-controlled pyrolysis reactor (see Fig. 1). The reaction temperature was maintained by an aluminium block (B) for temperatures below 550° C or a copper block for higher temperatures. The block was heated by a Nichrome-wire heater (I, 30 ohms), and temperatures were carefully controlled (±0·5° C) and recorded by a recording pyrometer (Brown Strip Chart proportional recording pyrometer) activated by a chromel - alumel thermocouple (C). (An extra thermocouple was included to activate an electrical cut-off switch to prevent melting of the aluminium block if over-heating occurred.) When the extent of the reaction was being followed by measuring the amount of unchanged ester or a high-boiling product, heating tape (not shown in Fig. 1) was wrapped around the short part of the column not inside the Dewar flask, which maintained the column temperature constant. The column temperature could be varied, but was always below pyrolysis temperatures. When only a quantitative determination of the light gases from pyrolysis (ethylene, carbon dioxide, etc.) was being made, as it was in a study made on the effect of substituents on the decomposition of ethyl-substituted benzoates, the higher-boiling products (substituted benzoic acids) were trapped in an ice - water trap before the gases were led into the chromatographic column.

Liquid samples, or solids dissolved in a suitable solvent, were introduced by a fixed-needle microsyringe through a Silastic septum (H) clamped rigidly to the reactor. Stripped Silastic rubber was employed to permit frequent changing of the septum when used at high temperatures. The reaction time could be controlled by regulating the helium inlet pressure and the gas flow at the exit of the column. A packing of Pyrex-glass beads was used in the reactor.

PROCEDURE—

After a suitable column packing has been chosen to attain the proper separation of the reaction products, introduce 2 to 10 μl of sample into the hot reactor at point H with a 50-μl fixed-needle syringe. Note the time of injection on the chromatographic chart. Qualitatively identify the products from the pyrolysis by comparing their retention times with retention times of known materials. Introduce the sample of the known material through the same injection port, H. Quantitatively determine the extent of pyrolysis by comparing the peak area of the unpyrolysed reactant with the area of a peak from a known amount of the reactant under carefully controlled conditions, *e.g.*, constant helium pressure and gas flow, filament current, column and reactor temperatures. Maintain the temperature of the reactor below pyrolysis temperature (but above condensation temperature) when determining the retention time and peak area for the unpyrolysed reactant itself. (If the stoicheiometry is known for the reaction, the extent of pyrolysis can be determined with reference to the peak areas of any of the products from the reaction.) Measure the peak areas with a planimeter unless an integrator, such as a Disc integrator (Disc Instrument Co., Santa Ana, California), is attached to the strip-chart recorder.

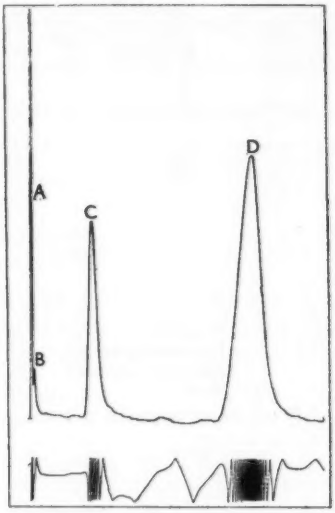


Fig. 2. Chromatogram of the products from the pyrolosis of *p*-methylphenylethyl carbonate: A, carbon dioxide; B, ethylene; C, *p*-cresol; D, unpyrolysed ester

Areas can also be compared with a mechanical digital counter of the Veedor Root type attached magnetically through a pulse convertor to the Disc integrator. The pulse convertor converts shaft rotations to digitals.

Compute all sample injections to millimoles before making quantitative comparison. Check the reproducibility by periodically repeating the pyrolysis of one compound chosen as a standard.

RESULTS

Fig. 2 is a chromatogram from the pyrolysis of 5μl of *p*-methylethylphenyl carbonate. The peaks were characterised as carbon dioxide, ethylene, *p*-cresol and unchanged ester. The temperature of the pyrolysis was 450° C; the products were separated on a 5-foot column packed with Silicon Dow 710 on a firebrick support at 200° C. The flow of helium was 55·4 ml per minute and the filament current on a Gow-Mac model 9346 thermal-conductivity cell was 150 mA. The extent of pyrolysis was determined by the amount of unchanged ester. On this column the carbon dioxide and ethylene were not completely separated. However, in the study of the pyrolysis

of ethyl benzoate, the extent of reaction was monitored by the amount of ethylene produced. The ethylene could be readily separated from the carbon dioxide (formed by the decarboxylation of the acid) on a column of 40- to 60-mesh silica gel. By careful injection of the sample and pyrolysing each sample five or six times, the extent of pyrolysis could be determined to within ± 2 per cent. Tables I and II show the type of quantitative results obtained by this technique. Several hundred analyses have been performed with this equipment within a few months on less than a millilitre of each ester.

TABLE I

PERCENTAGE DECOMPOSITION OF ARYLETHYL CARBONATES AT 500° C

$ArOCO_2C_2H_5$	Ortho, %	Para, %	Meta, %
CH_3O–	19	20	16
CH_3–	18	16	14
Cl–	12	13	13

TABLE II

PERCENTAGE DECOMPOSITION OF SUBSTITUTED BENZYLETHYL CARBONATES AT 525° C

$ArCH_2OCO_2C_2H_5$	Ortho, %	Para, %	Meta, %
CH_3–	23	26	25
Cl–	21	24	23

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THE COLORIMETRIC DETERMINATION OF COPPER IN SEA WATER SOLUTIONS

FOR the daily determination of microgram amounts of copper leached by sea water from large numbers of test panels of anti-fouling paint, we required a method that (*a*) was applicable in the presence of magnesium, calcium and chloride and (*b*) did not involve extraction of a coloured copper complex into a non-aqueous solvent. Concentrations of about 0·02 to 1 μg of copper per ml had to be determined, and an error of ± 10 per cent. was acceptable. Two methods[1,2] have been evaluated for use in these conditions. The procedures suggested by Riley and Sinhaseni[3] and Loveridge and his co-workers,[4] although more sensitive, require an additional operation to concentrate the extract.

Gran[1] proposed the use of oxalyldihydrazide, which forms a complex with copper in the presence of ammonia and acetaldehyde; he reported that the reagent was highly sensitive and selective and that the complex was formed in the presence of reasonable excess concentrations of chloride.

Jonassen, Chamblin, Wagner and Henry[2] described the use of disodium ethyl bis(5-tetrazolylazo)acetate (TETRA), which forms a complex with copper in the pH range 5 to 8. The complex is stable for long periods, and its colour obeys Beer's law over a wide range of concentrations, provided that a correction is applied to compensate for the colour of excess of the reagent.

The concentration of ammonia suggested by Gran as necessary for formation of the complex produced a heavy precipitate of hydroxides, which gave rise to serious interference with the measurement of optical density. However, formation of this precipitate could be suppressed by decreasing the concentration of ammonia used or by forming the complex in the presence of a mixture of ammonia and an ammonium salt, *e.g.*, the chloride or citrate. Ammonium chloride was the most suitable because it could be obtained in a high state of purity.

Gran reported that, for solutions in distilled water, maximum optical density was attained after 30 minutes, but when sea water solutions and the modified method were used, a longer time was required for maximum development of colour. In the presence of ammonium chloride or citrate, maximum colour was attained in 1 hour, and in the absence of an ammonium salt and with a decreased concentration of ammonia, it was necessary to set the solution aside for 5 hours. When formed under the various experimental conditions, the complex was stable for different periods, but always for more than 6 hours.

Jonassen, Chamblin, Wagner and Henry's method[2] was easily adaptable for use in sea water solutions. Control of the pH of such solutions did not appear to be critical, and no adjustments were necessary, although a slight increase in sensitivity was achieved when the pH of the sea water was decreased to 5·8 by means of a sodium acetate - acetic acid buffer solution. The complex was formed immediately, simply by adding the solution of TETRA to the test solution, and was stable for at least 24 hours.

Experimental

A test solution containing 1 p.p.m. of copper ion in sea water was used throughout; before preparation of this solution, the sea water was filtered to remove suspended solids.

Reagents—

The reagents used for determinations with oxalyldihydrazide were prepared as described by Gran[1] and those for determinations with TETRA as described by Jonassen, Chamblin, Wagner and Henry.[2] The 10 per cent. w/v solution of ammonium citrate used was prepared by dissolving the necessary amount of the laboratory-reagent grade salt in diluted (1 + 1 v/v) ammonia solution, sp.gr. 0·880. The ammonium citrate was found to give a colour reaction with oxalyldihydrazide under the test conditions; before use, therefore, it was extracted with a solution of dithizone in carbon tetrachloride until free from colour-producing impurities. The ammonium chloride solution used contained 5 per cent. w/v of the analytical-reagent grade salt in the diluted ammonia solution. Tests showed that this reagent contained no interfering impurities.

Comparison between methods—

Details of the procedures used and the results obtained are summarised in Table I, which also shows the sensitivities of the methods. Optical-density measurements were made in matched 4-cm cells with a Spekker absorptiometer fitted with Ilford No. 5 filters (550 mμ).

Table I

Experimental details and results

Method used*	A	B	C	D	E
Copper solution present, ml	10	10	10	10	10
Saturated oxalyldihydrazide solution present, ml	2	2	2	2	Nil
Ammonia solution, sp.gr. 0·880, diluted (1 + 1) present, ml	10	2	Nil	Nil	Nil
Ammoniacal 5 per cent. ammonium chloride solution present, ml	Nil	Nil	10	Nil	Nil
Ammoniacal 10 per cent. ammonium citrate solution present, ml	Nil	Nil	Nil	10	Nil
Aqueous actaldehyde solution (40 per cent. v/v) present, ml	10	10	10	10	Nil
TETRA solution (0·0001 M) present, ml	Nil	Nil	Nil	Nil	5
Distilled water present, ml	Nil	8	Nil	Nil	17
Total volume of solution, ml	32	32	32	32	32
Colour-development period, hours	0·5	6	1	1	Nil
Mean value found for molar extinction coefficient ($\times 10^{-4}$)	1·830	1·677	2·124	2·058	1·106
Coefficient of variation, %	2·0	4·1	2·2	1·1	0·86
Number of determinations	4	4	9	4	4

* The methods used were—

A. Gran's original method[1]; test solution contained 1 p.p.m. of copper in distilled water.
B. Modification of Gran's method; concentration of ammonia decreased.
C. Modification of Gran's method; ammonium chloride present.
D. Modification of Gran's method; ammonium citrate present.
E. Jonassen, Chamblin, Wagner and Henry's method.[2]

Interference studies—

The effect of the presence of other metal ions on the sensitivities of the methods was examined. Optical-density measurements were made on solutions of the copper - oxalyldihydrazide and

copper - TETRA complexes also containing an equal concentration or a 10- or 100-fold excess of a second metal ion; the results are shown in Table II. In these experiments, the copper - oxalyldihydrazide complex was formed in the presence of ammonium chloride (method C in Table I), as this procedure was considered to be the most satisfactory of those examined.

TABLE II

EFFECTS OF OTHER METALS

A solution containing 1 p.p.m. of copper in sea water was used in these experiments

Second ion added	Effect* on oxalyldihydrazide method when ratio of copper to second ion was—			Effect* on TETRA method when ratio of copper to second ion was—		
	1 to 1	1 to 10	1 to 100	1 to 1	1 to 10	1 to 100
Cd^{2+}	N	N	N	N	N	I
Co^{2+}	N	I	I	I	I	I
Cr^{3+}	I	I	P	N	I	I
Fe^{2+}	N	P	P	I	P	P
Fe^{3+}	N	P	P	N	P	P
Hg^{2+}	N	N	N	I	I	I
Mn^{2+}	N	I	P	N	N	N
Ni^{2+}	N	N	I	I	I	I
Pb^{2+}	N	N	P	N	N	N

* N = no interference; I = interference; P = formation of precipitate or turbidity.

CONCLUSIONS

The experiments described in this Note show that both oxalyldihydrazide and TETRA can be used for determining copper in sea water media, but the oxalyldihydrazide method as described by Gran[1] requires modifications. These modifications do not decrease the sensitivity of the method; indeed, our results show that the modified procedures are more sensitive.

Oxalyldihydrazide is a more sensitive reagent than TETRA and is less subject to interference from other metal ions. However, as can be seen from Table I, the application of TETRA is more simple, and this reagent can be made the basis of a rapid method giving an accuracy acceptable for our purpose.

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CO-PRECIPITATION OF COBALT WITH CHROMIUM AND ALUMINIUM HYDROXIDES IN QUALITATIVE ANALYSIS

THE loss of an element by co-precipitation with an earlier group is particularly important in semi-micro qualitative analysis. Large amounts of cobalt are brought down with aluminium and, especially, chromium in the hydroxide group under conditions recommended in several schemes of analysis. That co-precipitation has taken place is usually evident from the colour of the precipitates; aluminium hydroxide appears pink instead of white, and chromium hydroxide is red-brown (when spun in a centrifuge it gives a densely packed mauve precipitate) instead of grey-green. Such colour changes may, however, be partly or completely masked by the presence of other members of the hydroxide group.

Cobalt-60 is a radioactive isotope of cobalt. Its radiation (β and γ) can readily be detected and measured and permits co-precipitation of the element with the hydroxide group to be rapidly and easily determined. Accordingly, aluminium and chromium hydroxides were precipitated, under different conditions, in the presence of labelled cobalt, and the fate of the cobalt was ascertained by measurement with a γ-scintillation counter.

Cobalt is completely precipitated with chromium hydroxide, by 9 N ammonia solution, in the presence of 5 per cent. w/v of ammonium chloride, unless the ratio of cobalt to chromium exceeds about 3·5 to 1. Dilution of the reagent improves matters somewhat, but no significant amount of cobalt remains in solution (ratio of cobalt to chromium about 2 to 1) until the concentration of ammonium hydroxide used is below approximately 3 N; on the other hand, co-precipitation with aluminium hydroxide is less serious. The effect of the concentration of ammonium chloride is shown in Fig. 1. The pH of the solution at the termination of precipitation is also important when relatively concentrated ammonia solution is used and should be as low as possible, certainly below 6·3. This suggests that homogeneous precipitation might be advantageous; the results in Fig. 2 show that this is so.

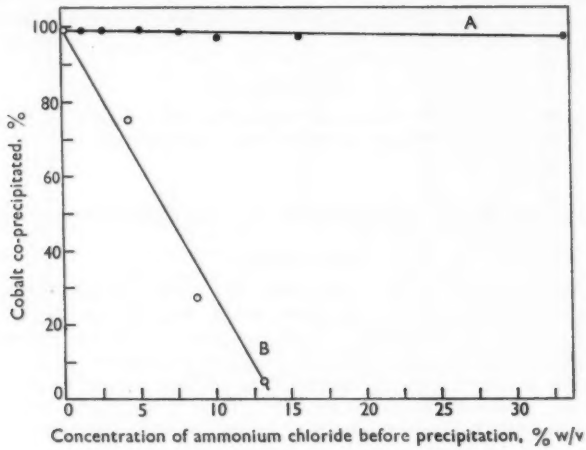

Fig. 1. Effect of concentration of ammonium chloride on amount of cobalt co-precipitated. Precipitation with 4·5 N ammonium hydroxide in presence of 2·09 mg of cobalt of: curve A, 0·99 mg of chromium; curve B, 1·19 mg of aluminium

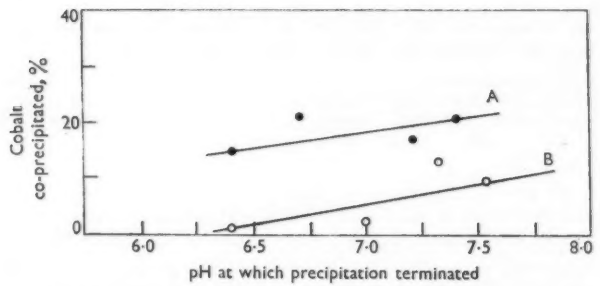

Fig. 2. Homogeneous precipitation through hydrolysis of urea. Precipitation in presence of 4·5 per cent. w/v of ammonium chloride and 2·09 mg of cobalt of: curve A, 0·99 mg of chromium; curve B, 1·19 mg of aluminium

Further experiments indicated that the co-precipitation of cobalt with chromium hydroxide is hardly affected by the presence of nickel, manganese, calcium, barium or fluoride, although it is appreciably reduced by zinc. With aluminium hydroxide, zinc reduces and fluoride almost eliminates co-precipitation of cobalt; however, it is the normal procedure in qualitative analysis to remove fluoride before precipitating the hydroxide group.

Washing the chromium or aluminium hydroxides with water or ammonium chloride solution removed only a small amount of cobalt. On the other hand, solution of the precipitate in 2 N

hydrochloric acid and then re-precipitation with 9 N ammonium hydroxide reduced the amount of cobalt carried down by 30 per cent.

Co-precipitation of cobalt was found not to be serious with ferric hydroxide and almost non-existent with the hydroxides of beryllium, cerium, lanthanum and zirconium.

Conclusion

It is evident that co-precipitation of cobalt with chromium hydroxide in semi-micro qualitative analysis presents a considerable problem. It is reasonable to assume that at least 0·1 mg of cobalt is required for detection and identification in its group; considerably more cobalt than chromium must be present in the original sample for this to be achieved. Co-precipitation with aluminium hydroxide, however, is not serious and can easily be reduced by the presence of sufficient ammonium chloride (see Fig. 1).

The use of sufficiently dilute ammonium hydroxide as precipitant appears to provide a remedy, but usually results in too large a volume of solution for convenience. An alternative is to dissolve and re-precipitate the hydroxides. However, the only satisfactory method appears to be to precipitate the group homogeneously as, for example, by the hydrolysis of urea. This may be carried out simply and quickly by the procedure described below.

After removal of hydrogen sulphide from solution and oxidation with concentrated nitric acid, the mixture is heated in a water bath, and 3 N ammonium hydroxide is added until the hydroxide precipitate just begins to form. Approximately 1 g of urea is added, and the water in the bath is maintained at boiling-point. Further amounts of urea are added as necessary, and the reaction is allowed to continue until the mixture is neutral to litmus paper and the precipitated hydroxides have settled. The whole procedure requires about 20 to 25 minutes.

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An Apparatus for Precipitating Metal Sulphides and Thiocarbonates with Potassium Trithiocarbonate as the Reagent

As reported in earlier papers,[1,2] potassium trithiocarbonate reagent has proved much superior to thioacetamide[3,4,5] as a substitute for hydrogen sulphide in the analysis of the inorganic cations. Further investigations have been carried out to devise improved methods of preparing and using the reagent, and this Note describes its preparation directly from sulphur, whereas the earlier method required use of hydrogen sulphide; the method, as standardised, improves the stability, purity and efficiency of the reagent. Further, a compact apparatus is described, which has been designed to accomplish the technique of "precipitation in homogeneous solution," so essential for the clean separation of cations. In this technique the precipitating agent is not added as such, but is generated in the solution; for potassium thiocarbonate reagent, either thiocarbonic acid or hydrogen sulphide is generated, depending on the pH of the solution.

Preparation of potassium trithiocarbonate reagent from sulphur—

The use of hydrogen sulphide is not desirable, nor is it capable of giving a pure product. The best method involves the use of sulphur. To prepare 100 ml of solution containing about 7 per cent. of potassium thiocarbonate reagent, shake 1·60 g of crystalline sulphur with 3 ml of purified carbon disulphide, sp.gr. 1·26. Dissolve 5·6 g of potassium hydroxide pellets in 90 ml of distilled water. Add the solution of alkali dropwise to the solution of sulphur in carbon disulphide, with constant shaking; an orange-red colour gradually develops owing to the formation of potassium trithiocarbonate. The sulphur solution must not be added to the potassium hydroxide solution nor must the latter be added in bulk to the sulphur solution or potassium carbonate may also be formed in accordance with the equation—

$$3CS_2 + 6KOH = K_2CO_3 + 2K_2CS_3 + 3H_2O.$$

If potassium carbonate has not been formed the pH of the reagent solution will be 9·5. The presence of CO_3^{2-} ions is detrimental to the clean separation of the "thiocarbonate group" of the

proposed scheme[2] of analysis and also undermines the stability of the reagent. The reagent solution is most conveniently protected from atmospheric carbon dioxide by storage under liquid paraffin rather than in an atmosphere of nitrogen.

APPARATUS AND TECHNIQUE—

Besides trapping any evolved toxic gas from precipitation reactions, the need for automatic stirring and control of pH, so as to ensure homogeneous precipitation with the minimum amount of the reagent, was desirable. The apparatus shown in Fig. 1 satisfies these requirements. The precipitation tube, P, is made from a Pyrex-glass boiling-tube, 6 inches long. To one side of this tube and at a point ½ inch from the bottom is attached a side-tube, S, 3 inches long and ¼ inch diameter, provided with a small bark cork. On the other side of tube P at a point 1½ inches below its mouth is an inclined exit tube connected to a filter pump by way of a large conical flask containing a 5 per cent. solution of potassium hydroxide; the conical flask is fitted in the manner of a wash-bottle. The upper end of the long tube of the conical flask is bent downwards for connection to the exit tube from P by means of rubber tubing. This prevents entry into the potassium hydroxide solution of any material overflowing from the tube P when its contents are poured out after precipitation. P is kept clamped during use, so that it can also be directly heated if required; it can be lodged in a wooden stand for carrying out reactions in the cold.

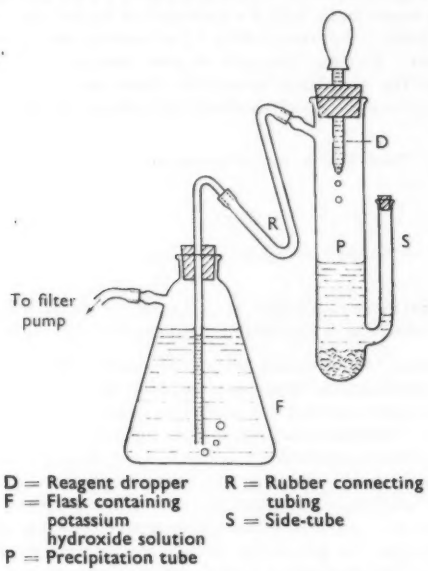

Fig. 1. Precipitation apparatus

The reagent solution is kept in a broad glass tube 3 inches long and lodged in a wooden stand. The solution is withdrawn from under the protective layer of liquid paraffin and then added to the test solution by means of a glass dropper fitted into a rubber cork. The dropper tube is about 4 inches long and is fitted in the cork so that, when placed in the reagent tube, its lower narrow end touches the bottom. This facilitates the withdrawal of the last drops of reagent solution.

METHOD OF OPERATING THE PRECIPITATION TUBE—

For precipitating the cation groups, place the test solution in tube P to a depth of not more than 2½ inches; keep side-tube S corked so that the test solution does not enter it. To achieve a cleaner separation of group-2 cations, as sulphides and thiocarbonates, maintain an optimum pH of 0·5 to 0·7 (check with a strip of methyl violet indicator-paper in side-tube S). Fill the dropper with potassium thiocarbonate reagent solution, and fit the cork tightly in the mouth of tube P. Start the suction pump; drops of thiocarbonate reagent solution will be drawn out of the dropper and will mix with the test solution. Remove the cork from side-tube S to admit air; the addition

of reagent will stop and the test solution and precipitate will be stirred as air bubbles through. This leads to homogeneous precipitation of the cations at constant pH, which rises only when all group-2 cations have been precipitated. Test for complete precipitation by switching off the pump; the precipitate will settle in a highly coagulated form leaving a clear liquid above. Make the clear liquid rise in the side-tube S by pressing the rubber tubing; a change in the indicator paper from violet to green indicates complete precipitation. Further addition of the reagent gives a bluish tint. Again apply suction to empty the side-tube S, and insert the cork. Finally remove the dropper and switch off the suction pump. Empty the contents of tube P for filtration.

The residue is again put into the tube P for further treatment. Group-5 metals of the proposed scheme[2] are precipitated likewise, but above pH 9·5 and as thiocarbonates. It may be noted that application of suction serves three purposes. First, it helps in trapping any hydrogen sulphide generated, secondly, it helps in the automatic addition of the reagent in drops and, thirdly, in stirring the contents during the precipitation. The hydrogen sulphide trapped in the potassium hydroxide solution can be used in the subsequent preparation of the thiocarbonate reagent.

I thank Professor T. R. Seshadri, F.R.S., Head of the Department of Chemistry, Delhi University, for providing necessary facilities for carrying out this work.

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ABSORPTIOMETRIC METHODS FOR THE DETERMINATION OF BORON

We have studied the most interesting paper by Lima, Pagano and Schneiderman[1] describing a statistical investigation into spectrophotometric determinations of boron. In this work the authors have employed two temperature levels, *viz.*, 100° and 55° C, for the drying operation; the latter value was chosen, at least partly, because of its use in our own work.[2] We think that this choice is unrealistic, since, in our own work, constancy of weight was not used as the criterion for the completion of the drying operation, and, moreover, the authors' procedure differs in several respects from that recommended by ourselves, *e.g.*, we did not use trichloroacetic acid. We are sure that the authors will agree that their conclusions must be confined to their own procedure only, and we wish to avoid any misapplication to our own work.

The following results show the influence of drying temperature on optical-density readings obtained by our method—

Drying temperature, °C	55	80	99 (approximately)
Corrected optical density (1-cm cell)	0·484, 0·503	0·095, 0·090	0·007, 0·031

These results were obtained by adding 1 ml (equivalent to 5 μg of boron) of a standard solution of boron to 5 ml of 0·1 N sodium hydroxide and 40 ml of methanol contained in a platinum dish. After evaporation to dryness over a beaker of nearly boiling water, 2 ml of oxalic acid - hydrochloric acid solution (6 per cent. w/v in 20 per cent. v/v hydrochloric acid) and 5 ml of curcumin solution (0·025 per cent. w/v in ethanol) were added, and the solution was evaporated on a specially prepared water bath set to the required drying temperature. Heating was continued for 30 minutes after the odour of hydrochloric acid had disappeared. The residue was extracted in 50 per cent. v/v aqueous acetone and diluted to 50 ml with the same solvent, and the optical density measured in 1-cm cells with a Spekker absorptiometer fitted with a mercury-vapour lamp and Ilford 605 and H503 filters.

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Book Reviews

PURE FOOD AND PURE FOOD LEGISLATION. Edited by A. J. AMOS, B.Sc., Ph.D., F.R.I.C. Pp. xii + 167. London: Butterworths Publications Ltd. 1960. Price 21s.

This book contains the full texts of the eight papers read at the Pure Food Centenary Conference, held at the Royal Institution on September 21st to 23rd, 1960.

After a short preface by Dr. Amos and the formal opening of the Conference by Lord Waldegrave, the first paper, by Dr. Hamence, carries us through the early years of the pure food campaign, showing the significance of the first general Act of 1860, and traces the gradual transition to more effective control. A tribute is paid to the pioneer work of Public Analysts, many of whose names are deservedly famous in the history of food chemistry.

The second paper, by Dr. Hughes, is an admirable exposition of the place of research in the factory and the importance of careful scientific control at every point in manufacture and distribution. Dr. Hughes stresses the need for long-term fundamental research and discusses the work of the D.S.I.R. and of the trade research associations that receive annual grants from that Department. The important reports on food additives of the Preservatives Sub-Committee of the Food Standards Committee are referred to, as are the functions and work of the Food Group of the Society of Chemical Industry. It is clear from Dr. Hughes' remarks that the utmost care is taken by all reputable manufacturers in the preparation and packaging of foods, but consumers who have suffered from too sanguine an estimate of keeping quality in some retail shops may wish that he had gone more fully into the vexed question of shelf-life and dating of packages, which was raised in the discussion.

Dr. Malloch's paper is mainly concerned with the problems arising from the immense volume and variety of the food trade between the different parts of the Commonwealth and the difficulties encountered in the production and distribution of enough food of good quality in under-developed countries.

Dr. Norman Wright points out that food and its related products form over one third of the total amount of international trade, expressed in monetary terms. He discusses the prospects of, and the obstacles to, international agreement on food legislation and control, with the object of easing the difficulties of importers and exporters faced with totally dissimilar sets of regulations in almost every country. Apparently, a successful start has been made in this tremendous task, first by establishing a clearing-house and information centre and secondly by drawing up regional Codices of general principles, *e.g.*, the basic criteria that should govern the use of food additives.

The discussions on all these papers are of great interest and are given in full.

Dr. Morrell, Professor Reuter and Dr. Larrick deal, respectively, with national problems in Canada, Australia and the United States. The first named makes some pertinent remarks on the question of advertising, a subject of increasing concern in this country, with the advent of commercial television. The housewife may easily be induced by advertisement to spend an undue proportion of the weekly budget on comparatively worthless foods. Professor Reuter gives an account of the differences that arose between Commonwealth and States governments in Australia, barring the way for so many years to agreement on food legislation and control. Dr. Larrick, whose paper was read in his absence by Mr. Harvey, makes some surprising disclosures as to the number of chemical food additives already sanctioned for use in the United States. He also discusses the problem of nutritional quackery, the cost of which to the consumer is estimated at about 500 million dollars annually. The contamination of food by agricultural pesticides is also an acute problem in the United States, and Mr. Harvey's remarks gave rise to a lively discussion.

The series is completed by Professor Frazer, with a paper on the integration of food research. He stresses the importance of ensuring that, when any problem involving public health arises, the combined weight of knowledge that in any way relates to it, agricultural, chemical, physical, biochemical, pharmacological, toxicological, bacteriological and clinical, can readily be mobilised for its solution.

Dr. Amos and his executive committee are to be congratulated on the publication of these valuable papers, which form a worthy record of a well-conceived and well-organised centenary celebration.

G. W. MONIER-WILLIAMS

METHODS IN GEOCHEMISTRY. Edited by A. A. SMALES and L. R. WAGER, F.R.S. Pp. viii + 464. New York and London: Interscience Publishers Inc. 1960. Price 94s.; $13.50.

As seen by V. M. Goldschmidt, the task of the geochemist is to determine quantitatively the composition of the Earth in all its parts and then to discover the laws that condition the

distribution of individual elements. To this end the geochemist—and the cosmologist, whose interests are similar, but necessarily more extensive—will take advantage of every possible analytical technique. Because so many of these are now highly specialised, it cannot be expected that any one worker will feel equally at home with them all, and for this good reason the editors have chosen a distinguished team of specialists to describe their respective fields and techniques.

An essential preliminary to a significant analytical determination is the preparation of a truly representative sample that can be presented to the analyst in its natural state, free from adventitious contamination and unaltered by loss of any of the components. This important topic is admirably dealt with (28 pp.) by Professor Wager and Dr. G. M. Brown. Dr. Vincent then deals with classical gravimetric and volumetric methods of determining major constituents, with flame photometry for sodium and potassium, and with colorimetry (absorptiometry), turbidimetry and fluorimetry for trace elements (48 pp.).

In the following chapters various physical methods of analysis are described, *viz.*, Spectrochemical Analysis (Ahrens and Taylor, 30 pp.), X-Ray Fluorescence (Shalgosky, 37 pp.), Mass Spectrometry and Stable Isotope Geochemistry (Mayne, 54 pp.), Mass Isotope Dilution Analysis (Webster, 45 pp.), Radio-chemical Methods (Moorbath, 50 pp.), Radioactivation Analysis (Mapper, 61 pp.) and Polarography (Moorbath, 31 pp.). Although Smales' name does not appear as the author of any particular chapter, his influence is strongly felt throughout, not least in the final stimulating chapter on Modern Chemical Separation Methods (Cornish, 37 pp.), which deals synoptically with ion-exchange resins, chromatographic techniques, solvent extraction and paper chromatography.

The impression throughout this book is of practical analysts writing about their own experiences. In every case, care is taken to indicate the range of application of a particular technique, its reliability, its advantages and its limitations. The bibliographies are extensive, well chosen and up to date; the index is adequate. Great credit reflects on the editors in having achieved so homogeneous a book, considering the number of their collaborators and the variety of topics covered, although their task must have been lightened by the close contacts established over the years between the staffs (present and past) of the Department of Geology and Mineralogy at Oxford and the Analytical Chemistry Division of A.E.R.E., Harwell, when carrying out fundamental original work in geochemistry.

H. IRVING

TOXIC PHOSPHORUS ESTERS: CHEMISTRY, METABOLISM AND BIOLOGICAL EFFECTS. By RICHARD D. O'BRIEN. Pp. xii + 434. New York and London: Academic Press Inc. 1960. Price 103s. 6d.

This book mainly deals with the biochemistry and physiological properties of organo-phosphates. The compounds discussed are those widely used in commerce as insecticides and, as such, are of great interest to analytical chemists.

The outstanding characteristic of these compounds is their ability to inhibit the cholinesterase enzyme systems of living organisms, and, in consequence, the greater part of the book deals with this anticholinesterase behaviour, both in the living organism and in isolated enzyme systems *in vitro*. The mechanism of nerve transmission is described, together with the effects of poisoning by these compounds. Protection against poisoning and therapy after poisoning are discussed. The metabolic break-down of the insecticides both in animals and vegetables is given very full treatment.

The section on the analysis of animal and plant products for residues of the organo-phosphorus insecticides is briefly but well presented. Inherent difficulties and practical recoveries are discussed for some materials. The bibliography of this section does not include methods published since 1959 and only a few since 1958, but is otherwise good. The author gives a brief account of the application of infra-red and ultra-violet spectrography and of nuclear magnetic resonance to the identification of organo-phosphorus insecticides and includes a short discussion of the use of bond-refraction constants to deduce the refractive index of a compound. Paper chromatography receives mention, but is not discussed at any great length. Column chromatography also is mentioned, particularly with reference to subsequent infra-red identification of the insecticides. Gas - liquid partition chromatography as an analytical tool does not receive mention, presumably as the first papers on the application of this technique to organo-phosphorus insecticides were published after the compilation of the bibliography. The analytical section closes with a description of the methods used for measuring the anticholinesterase effects of the insecticides.

Appendix I deals with the electronic interpretation of the structures of these substances, and Appendix II lists the names, manufacturers and structures of the compounds mentioned in the text.

This is a very useful list and will greatly help the analyst in his work. Although this book is not essential for the proper analysis of organo-phosphorus insecticides, it forms a very interesting and informative background to the subject. The analyst who reads it will enlarge his knowledge, particularly with respect to the complexity of the animal and vegetable systems with which he must work if residual amounts of the compounds are to be assessed. It may also serve to bring into perspective the quantitative aspect of the subject and to emphasise the difficulties of complete recovery of the insecticides at low residue levels. The book is well presented, and the references, tables and index are satisfactory.

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The Radiochemistry of Ruthenium. By E. I. Wyatt and R. R. Rickard. Pp. viii + 78. Washington, D.C.: National Academy of Sciences—National Research Council. 1961. Price $1.00.
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REPRINTS OF "THE TOXICOLOGY OF NITRATES AND NITRITES WITH PARTICULAR REFERENCE TO THE POTABILITY OF WATER SUPPLIES" BY E. H. W. J. BURDEN

Reprints of the Review Paper, "The Toxicology of Nitrates and Nitrites with Particular Reference to the Potability of Water Supplies," by E. H. W. J. Burden, published in this issue of *The Analyst* (pp. 429–433), will be available shortly from the Assistant Secretary, The Society for Analytical Chemistry, 14 Belgrave Square, London, S.W.1, at 2s. 6d. per copy, post free. A remittance for the correct amount, made out to The Society for Analytical Chemistry, must accompany the order; these reprints are not obtainable through Trade Agents.

Errata

February (1961) issue, p. 113, 13th line under "Reagents." *For "Ammonia solution, 50 per cent. w/v" read "Ammonia solution*—Ammonia solution, sp. gr. 0·880, diluted (1 + 1)."

March (1961) issue, p. 200, 28th and 29th lines (the acknowledgments). *Delete* all words after "Note."